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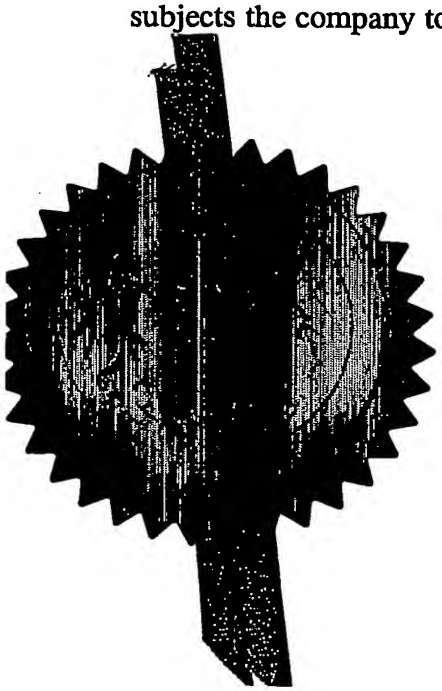

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K.U.Leuven Research and Development - Groot Begijnhof 59 - 3000 Leuven
Represented by Dr. Ivo Roelants, IPR Officer
Patents ADP number *(if you know it)* 07665649001

If the applicant is a corporate body, give the country/state of its incorporation Belgium

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Integrase Tetramers

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INTEGRASE TETRAMERS

FIELD OF THE INVENTION

Present invention includes a mammalian cellular protein that associates with integrase (integrase interacting protein), and antibody or antisense inhibition of said integrase interacting protein. The novel integrase interaction protein is a target for HIV replication prevention or inhibition.

SUMMARY OF THE INVENTION

In a study of HIV-1 integrase (IN) complexes derived from nuclei of human cells stably expressing the viral protein from a synthetic gene it was demonstrated that in the nuclear extracts IN exists as part of a large distinct complex with apparent Stokes radius of 61 Å, which dissociates upon dilution yielding a core molecule of 41 Å. The IN complexes were isolated from cells expressing FLAG-tagged IN. By present invention it was demonstrated that the 41 Å core is a tetramer of IN, whereas 61 Å molecules are composed of IN tetramers associated with a cellular protein with an apparent molecular weight of 76 kDa. This integrase interacting protein (Inip76) was found to be identical to LEDGF/DFS70/p75, a protein implicated in regulation of gene expression and cellular stress-response. HIV-1 IN and Inip76 co-localized in the nuclei of human cells stably expressing IN. Furthermore, it has been demonstrated by present invention that recombinant Inip76 strongly promoted strand-transfer activity of HIV-1 IN in vitro. Our findings reveal that the minimal IN molecule in human cells is a tetramer and and clearly demonstrates that Inip76 is likely to play a role in retroviral integration.

BACKGROUND OF THE INVENTION

Establishment of the provirus, a DNA copy of the viral genome integrated into the host cell chromosome is an obligatory step in retroviral replication. Stable integration into the human genome is one of the primary reasons for the persistence of the human immunodeficiency virus (HIV) infection, which leads to the acquired immunodeficiency syndrome (AIDS). Therefore, HIV IN, the enzyme orchestrating the insertion of the DNA replica of the viral genome into the cellular chromosomal DNA, is a potential target for antiretroviral therapy. The recent discovery of the new lead compounds able to protect cells against HIV infection by inhibiting integration raises hope that integrase inhibitors will be added to future combination cocktails (for reviews see Debyser et al., 2002; Miller and Hazuda, 2001; Pani and Marongiu, 2000; Pommier et al., 2000)).

Mechanistically and structurally, retroviral integrases (reviewed in Asante-Appiah and Skalka, 1997; Brown et al., 1987; Craigie, 2001; Hindmarsh and Leis, 1999) are similar to the well-studied prokaryotic Mu and Tn5 transposases and belong to a family of DNA strand transferases that catalyze DNA strand cleavage/ligation via direct trans-esterification. In the course of viral integration, HIV IN performs two enzymatic reactions: first is the removal of the 3'- GT dinucleotides from the long terminal repeats (LTRs) (i.e. the 3'-processing reaction). The second reaction is the insertion of the recessed 3'- viral DNA ends into the opposite strands of the target DNA wherein the 3' hydroxyls of the processed LTR ends attack two phosphodiester bonds in the target DNA molecule (the strand-transfer reaction). Insertion of the two viral LTRs takes place in a coordinated fashion across the major groove of the target DNA. As a result, the integrated provirus is flanked by two 5-nucleotide gaps as well as two mismatched 5'-AC dinucleotides, which are probably repaired by cellular enzymes (Yoder and Bushman, 2000). HIV IN, like other retroviral integrases, is comprised of three structural domains. The amino terminal domain contains the HHCC zinc finger motif, coordinates Zn and appears to be important for formation of the active IN multimers. The central, catalytic or "core" domain (residues 50-212) contains the active site (i.e. the DDE motif composed of D64, D116 and E152) and is structurally very similar to the catalytic domains of Mu

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phage and Tn5 transposases. In the context of the full-length enzyme, the core domain is thought to make sequence-specific contacts with the DNA. The carboxy terminal fragment possesses unspecific DNA-binding activity and is critical for multimerization of the enzyme. All single domain fragments of IN are dimeric when produced separately; the full-length HIV IN as well as other retroviral integrases form multimers under the conditions of the in vitro enzymatic assays (Ellison et al., 1995; Engelman et al., 1993). Recombinant HIV and avian sarcoma virus (ASV) integrases have been shown to behave as mixtures of monomers, dimers and tetramers (Coleman et al., 1999; Deprez et al., 2000; Jenkins et al., 1996). The presence of octamers and larger complexes has been suggested by some studies (Lee et al., 1997; Leh et al., 2000). Virion-associated HIV IN was shown to be in an oligomeric form, whereby dimers and higher order multimers appeared to be stabilized by disulfides, although the complexes were not studied under native conditions (Petit et al., 1999). Earlier, IN derived from avian myoblastosis virus (AMV) particles was demonstrated to be multimeric, behaving at least as a dimer (Grandgenett et al., 1978).

The 3'-processing and strand-transfer reactions can be reproduced in vitro using recombinant enzyme preparations and DNA substrates that mimic LTR ends. *In vivo* retroviral integration is preceded by the assembly of a stable and compact preintegration complex (PIC), that contains a 9.5 kb linear DNA copy of the viral genome associated with viral and cellular proteins (Brown et al., 1987; Bukrinsky et al., 1993; Ellison et al., 1990; Farnet and Haseltine, 1991; Miller et al., 1997). Several cellular proteins have been suggested to play auxiliary roles during retroviral integration. Thus, the barrier-to-autointegration factor (BAF) has been reported to protect Moloney murine leukemia virus (MoMLV) PICs against suicidal self-integration (Lee and Craigie, 1998). Another cellular protein, HMG-I(Y) was found in HIV PICs and appeared to be essential for their integration activity in vitro (Farnet and Bushman, 1997; Miller et al., 1997). Conversely, BAF could substitute for HMG-I(Y) at least in vitro, partially restoring integration activity of salt-denatured HIV-1 PICs (Chen and Engelman, 1998). Yet, BAF remains to be shown to co-fractionate with retroviral PICs. Both BAF and HMG-I(Y) are small DNA-binding proteins able to bridge and deform DNA molecules (Reeves and Beckerbauer, 2001; Zheng et al., 2000). They are thought to play structural roles within retroviral PICs, possibly juxtaposing both LTRs. Similarly, Mu phage transposase as well as enzymatically-unrelated λ phage integrase require the DNA-bending host proteins IHF

and/or HU to form committed synaptic complexes (Friedman, 1992; Mizuuchi, 1992). IHF and HU appear to substitute for each other in promoting formation of the active λ integrase-DNA complexes in vitro (Segall et al., 1994). Another potential co-factor for HIV integration, the integrase interactor 1 (Ini1 or hSNF5/BAF47), was originally discovered in a yeast two-hybrid screen for human proteins interacting with HIV-1 IN (Kalpana et al., 1994). Ini1 is a subunit of the 2 MDa SWI/SNF chromatin-remodeling complex (Wang et al., 1996). It has been proposed that Ini1 plays a role during retroviral replication by directing the PICs to open chromatin regions (Kalpana et al., 1994) or by modulating expression of the integrated provirus (Turelli et al., 2001). Recent studies demonstrated that GFP-tagged Ini1 was exported from the nuclei of infected cells and co-localized with incoming sub-viral particles (Turelli et al., 2001). Ini1 has also been reported to enhance the release of infectious HIV particles; detectable amounts of Ini1 have been shown encapsulated within virions (Yung et al., 2001).

Using a synthetic gene, we have been able to achieve efficient expression of HIV-1 IN in human cells (Cherepanov et al., 2000). By present invention we have now characterized HIV-1 IN protein complexes present in nuclear extracts from cells stably expressing this viral protein. For the time we reported a HIV integrase-interacting protein that forms a distinct complex with IN in human cells and provided an insight into the oligomeric state of intracellular HIV IN, suggesting that the minimal cellular IN complex is a tetramer. It is an object of present invention to prevent or inhibit the integration of the HIV genome into the host cell chromosomes by inhibiting the interaction between the integrase interacting protein and integrase.

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ILLUSTRATIVE EMBODIMENTS OF THE INVENTION

Examples

Methods

Example 1 Recombinant DNA

The HIV-1 integrase expression constructs were based on the episomal pCEP4 vector (Invitrogen, Groningen, The Netherlands). The plasmid pCEP-IN°ala is identical to the published pCEP-IN° plasmid (Cherepanov et al., 2000) with the difference that the Gly codon in the second position of the synthetic open reading frame (ORF) was mutated to Ala. As a result, the construct expressed native HIV-1 IN with an addition of Met-Ala dipeptide at the N-terminus. To create the FLAG epitope-tagged IN expression construct pCEP-IN°alaFLAG, the IN synthetic ORF from pCEP-IN°ala was amplified in two consecutive steps with the sense primer 5'-GGCTAGATATCACTAGCAAC CTCAAACAG plus two anti-sense primers 5'-GTCGTCCTTGTAATCGCCGTCC TCATCTTGACGAGAG and 5'-GGCGCTCGAGTTACTTGTCATCGTCGTCCTTGT AATCGC, the resulting PCR fragment was digested with XhoI and cloned between the EcoRI (blunt) and XhoI sites of the pCEP4 vector. This plasmid expressed HIV-1 IN carrying the C-terminal FLAG epitope (DYKDDDDK). The plasmid pRP1012, for bacterial expression of His_6-tagged HIV-1 IN, was a gift of Dr. R. Plasterk (The Netherlands). To obtain pCP6H75, the plasmid used for bacterial expression of His_6-tagged Inip76/LEDGF, the PCR fragment amplified from a HeLa cDNA with the primers 5'-GGCCGGATCCGACTCGCGATTTCAAACCTGGAGAC and 5'-CCGCGAATTCT AGTTATCTAGTGTAGAATCCTTC was digested with BamHI and EcoRI and cloned between the BamHI and EcoRI sites of pRSETB (Invitrogen). To prepare the mini-HIV IN substrate, the plasmid pU3U5 (Cherepanov et al., 1999) was digested with ScaI.

Example 2: Cell Culture

The human embryonic kidney cells expressing SV40 large T antigen, 293T were obtained from Dr. O. Danos (Evry, France). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 20 µg/ml gentamicin at 37°C in 5% CO_2 humidified atmosphere. To

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establish stable cell lines, 293T cells were transfected by electroporation with integrase expression constructs and selected with 200 μg/ml of hygromycin B (Invitrogen). The selected cell lines, 293T-IN^{s}ala and 293T-IN^{s}alaFLAG, expressed IN at the levels similar to the previously reported 293T-IN^{s} cells (Cherepanov et al., 2000) as determined by western blotting and immunofluorescence. IN expression was stable for over 40 passages. For radioactive immunoprecipitation experiments cells were labeled in methionine/cysteine-free DMEM (Invitrogen) supplemented with 10% dialyzed FCS plus 0.1 mCi/ml of TRAN^{35}S-LABEL (ICN Biomedicals, Asse-Relegem, Belgium) for 24 hours.

Example 3: Preparation of Nuclear Extracts

293T-IN^{s}ala or 293T-IN^{s}alaFLAG cells grown to a confluency of 80-90% were dissociated from plastic dishes using trypsin, washed with PBS and re-suspended in the modified cytoskeleton buffer (10 mM Pipes [pH 6.8], 10% (w/v) sucrose, 1 mM DTT, 1 mM $MgCl_2$ plus the EDTA-free protease inhibitor cocktail (Roche, Brussels, Belgium)) (Fujita et al., 1997) containing 100 mM NaCl (referred to as 100CSK buffer). Cells were lysed for 10 min on ice with 0.5% NP40; nuclei were pelleted and washed with 100CSK. To extract IN, nuclei were re-suspended in the 400CSK buffer (same as 100CSK, but containing 400 mM NaCl) and left on ice for 5 min, the chromatin was removed by centrifugation at 7,500 rpm for 2 min. The total protein content of the nuclear extracts was measured using the BCA protein assay (Pierce, Rockford, IL), with bovine serum albumin (BSA) as the standard.

Example 4: Chemical Cross-linking

The nuclear extracts were diluted using 400CSK buffer to adjust the total protein concentration to 100, 20 or 4 μg/ml. DTSSP (Pierce, Erembodegem-Aalst, Belgium) was dissolved in water immediately prior to the experiment and used at 2, 0.5 or 0.1 mM. Cross-linking reactions were allowed to proceed for 15 min at room temperature, were terminated by addition of ¼ volume of the 4 x SDS sample buffer (200 mM Tris HCl [pH 6.8], 4% SDS and 40% (v/v) glycerol) and incubation at room temperature for 20 min.

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Example 5: Gel Filtration Chromatography

Nuclear extracts and immunopurified IN_f were fractionated on a Superdex 200 HR 10/30 gel filtration column (Amersham-Pharmacia). The 400CSK buffer was used in all chromatography experiments. The column was operated at 0.6 ml/min, 4°C, and calibrated using low and high MW gel filtration standards from Amersham-Pharmacia (blue dextran; thyroglobulin, R_s/Mw 85 Å/669 kDa; ferritin, 61 Å/440 kDa; catalase, 52.2 Å/232 kDa; aldolase, 48.1 Å/158 kDa; BSA, 35.5 Å/67 kDa; chymotrypsinogen A, 20.9 Å/25 kDa). The sample volume was kept at 200 µl; fractions of 300 µl were collected and analyzed by western blotting using polyclonal anti-IN antibodies. When necessary, gel filtration fractions were concentrated by precipitation with trichloroacetic acid. Stokes radii (R_s) and approximate MWs of the IN complexes were determined from their experimental partition coefficients (K_{av}) as described (Siegel and Monty, 1965).

Example 6: Western Blotting and Immunoprecipitation

The gradient 4-12 and 4-20% Novex Tris-Glycine gels were purchased from Invitrogen. Proteins were transferred onto PVDF membrane (Bio-Rad, Nazareth, Belgium); detection was done with ECL+ (Amersham-Pharmacia, Roosendaal, The Netherlands). The rabbit polyclonal anti-HIV-1 IN were home made and used at a final dilution of 1:30.000 (Cherepanov et al., 2000). The anti-FLAG M2 monoclonal antibody was from Sigma-Aldrich, the monoclonal anti-DNA-PKcs Ab-4 cocktail from NeoMarkers (Fremont, CA) and the monoclonal anti-LEDGF p75/p52 from BD Biosciences (Erembodegem, Belgium). The affinity purified anti-hMCM3 polyclonal antibody was a gift from Dr. Knippers R. (Universty of Konstanz, Germany). A combination of prestained MW markers (New England Biolabs, Hertfordshire, UK) and Mark12 (Invitrogen) was used to estimate MWs of the cross-linking products and Inip76. DNA PKcs detected in a 293T nuclear lysate sample using the anti-DNA PKcs Ab4 antibody served as the 470 kDa marker in some western blots.

In the initial immunoprecipitation experiments, 30 µl of protein G agarose (Roche) and 1-3 µg of the anti-FLAG M2 antibody was added to the nuclear extracts prepared in 400CSK and diluted to obtain total protein concentration of 200 µg/ml; the suspension was stirred at 4°C overnight (12-18 hours). The agarose beads were washed once with 400CSK and 4 times with 100CSK plus 0.1% NP40. Protein was eluted in 400CSK buffer by addition of 200 µg/ml FLAG peptide (Sigma-Aldrich) or in

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SDS PAGE sample buffer. To purifiy LEDGF-IN$_F$ complexes, immunoprecipitation was carried out using undiluted nuclear extracts (700 µg/ml total protein) for 3-5 hours. To identify the Inip76 protein by N-terminal sequencing and mass spectrometry, the procedure was upscaled. 293T-IN^{F}alaFLAG cells were grown to confluency on five 500 cm^2 dishes (VWR International, Leuven, Belgium), lysed with 0.5% NP40. IN complexes were extracted from the nuclear pellets into 13 ml of the 400CSK buffer and incubated with 300 µl of protein G agarose beads and 40 µg of the anti-FLAG M2 antibody for 4.5 hours. The IN$_F$ complexes were eluted in 700 µl of the 400CSK buffer with 200 µg/ml FLAG peptide.

Example 7 : Amino Terminal Sequencing and Mass Spectrometry

Immunopurified IN$_F$-Inip76 complexes were precipitated with trichloroacetic acid and redissolved in the SDS PAGE sample buffer. Approximately 3 µg of the Inip76 protein, electroblotted onto a Sequi-Blot PVDF membrane (Bio-Rad) from an SDS PAGE gel, was subjected to Edman degradation on a pulsed liquid phase Procise 491cLC protein sequencer (Applied Biosystems, Lennik, Belgium). For mass spectrometry analysis the coomassie blue-stained band of Inip76 was cut from an SDS PAGE gel, destained in a 200 mM ammonium bicarbonate/50% acetonitrile, air-dried and soaked in 8 µl trypsin solution (16 ng trypsin (Promega) in 50 mM ammonium bicarbonate) on ice for 20 min. Following overnight digestion at 37°C, the supernatant was recovered, and the gel slice was extracted twice using 60% acetonitrile/0.1% formic acid. The extracts and the supernatant were pooled and dried in a Speedvac concentrator. The peptides were re-dissolved in 0.1% formic acid and analysed by on-line nanoflow high performance liquid chromatography tandem mass spectrometry (LC/MS/MS) on an UltiMate capillary LC system (LC-Packings, Amsterdam, The Netherlands) coupled to a Q-Tof mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization source. Technical details of this system are reported elsewhere (Devreese et al., J. Chromatography A, in press). All spectra were processed using the MassLynx and MaxEnt software delivered with the mass spectrometer.

Example 8: Indirect Immunofluorescence Microscopy

293T-IN^{F}alaFLAG cells grown in Lab-Tek II glass chamber slides (VWR International) were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 10 min and

permeabilized in ice-cold methanol. The cells were further blocked in PBS supplemented with 20 mM ammonium chloride and 10% FCS and incubated with rabbit polyclonal anti-FLAG antibodies (diluted 1:10.000 in PBS/10% FCS) (Sigma-Aldrich) and monoclonal anti-LEDGF (1:300) or anti-DNA PKcs (1:300) followed by Alexa-555 anti-rabbit and Alexa-488 conjugated anti-mouse IgG antibodies (Molecular Probes, Leiden, The Netherlands). The nuclear DNA was labeled with 5 µM To-Pro3 iodide (Molecular Probes). Confocal laser scanning fluorescent microscopy and imaging was carried with an LSM510 system (Carl Zeiss, Zaventem, Belgium) using a 488 nm Ar ion laser with a 505-530 nm band pass filter for Alexa-488, a 543 nm HeNe laser with a 565-615 nm filter for Alexa-555 and a 633 nm HeNe laser with a low pass 650 nm filter for ToPro-3. All acquisitions were done in the multi-track mode.

Example 9: Recombinant proteins

The His_6-tagged HIV-1 IN was produced from the pRP1012 plasmid in the Endo I-free host Escherichia coli strain PC1 (BL21(DE3), Δ*endA*::Tc^R, pLysS) (Cherepanov et al., 1999). The protein was purified from the soluble fraction by chromatography on Ni-NTA agarose (Qiagen, Hilden, Germany) and Heparin Sepharose (Amersham Pharmacia, Uppsala, Sweden) in the presence of 7.5 mM 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS) (Sigma-Aldrich). The His_6-tag was removed by incubation of the purified protein with thrombin (Novagen). The His_6-tagged Inip76 was expressed from the plasmid pCP6H75 in PC1 by induction with 1 mM isopropylthiogalactopyranoside (IPTG) at 29°C for 3 hours in LB medium. The soluble protein was then purified by batch adsorption to Ni-NTA agarose and chromatography on a 1 ml HiTrap Heparin Sepharose column (Amersham Phamacia). The protein was eluted from the Heparin Sepharose column using a linear NaCl gradient in 30 mM TrisHCl [pH 7.0]. Peak fractions collected at approximately 800 mM NaCl were pooled and concentrated using Centricon-30 (Millipore, Brussels, Belgium) to obtain the final protein concentration of 1 mg/ml. The protein was estimated to be at least 95% pure by SDS PAGE.

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Example 10 Disruption of the nuclear and chromosomal localization of HIV IN using small interfering RNA molecules specific to the Inip76 mRNA.

RNA interference with synthetic short RNA duplexes has been used to knock-down expression of endogenous genes in mammalian cells (Elbashir et al., 2002). We used short interfering RNA (siRNA) duplexes to study the effect of depletion of Inip76 in 293T-INsalaFLAG cells on the distribution of HIV-1 IN

i): siRNA Preparation

All oligonucleotides for siRNA preparation were chemically synthetized by Dharmacon (CO, USA) and Xeragon (MD, USA) and were composed of ribonucleotides (A,U,G and C) plus a pair of 3'-terminal desoxy-thymidines (dTdT).

Small interfering RNA molecules (siRNA) were prepared by annealing the following pairs of oligonucleotides:

i) CAGCCCUGUCCUUCAGAGA-dTdT plus UCUCUGAAGGACAGGGCUG-dTdT, to obtain 76A RNA;

ii) AGACAGCAUGAGGAAGCGA-dTdT plus UCGCUUCCUCAUGCUGUCU-dTdT, to obtain 76B RNA;

iii) CAGAUGCAUUGAGGCCUUG-dTdT plus CAAGGCCUCAAUGCAUCUG-dTdT, to obtain 76C RNA;

iv) GCGCGCUUUGUAGGAUUCG-dTdT plus CGAAUCCUACAAAGCGCGC-dTdT, to obtain NC RNA.

The 76A, 76B and 76C siRNA molecules are thus designed to be specific for the Inip76 mRNA and contain an anti-sense strand that can hybridize to the target mRNA. The NC molecule is an RNA duplex which is not specific to Inip76 RNA nor to any other known human mRNA.

ii. Transfection of the 293T-INsalaFLAG Cells with the siRNA Molecules

293T-INsalaFLAG cells expressing FLAG-epitope tagged HIV-1 IN were seeded into 8 well Nunc LabTekII chamber glass slides (VWR International) and were transfected at a confluency of about 25% with each siRNA using GeneSilencer, a liposomal transfection reagent purchased from GTS (Gene Therapy Systems, CA, USA).

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Transfection was done according to the manufacturer's recommendations. Briefly, for transfection of each well, 0.3 µg of RNA and 1.75 µl of the GeneSilencer reagent were used to transfect each well of the slide.

Expression and distribution of IN and Inip76 was assessed by indirect immunofluorescence. Two days post transfection the cells were fixed with 4% formaldehyde in PBS for 5 minutes, followed by four washes in PBS and treated with ice-cold methanol for 5 minutes. Fixed and permeabilized cells were then incubated in blocking solution (10 % fetal calf serum/10 mM ammonium chloride in PBS) for 30 minutes at room temperature. The cells were incubated with mouse monoclonal anti-human LEDGF antibody (purchased from BD Bioscience, KY, USA) diluted 1:200 in blocking buffer or monoclonal anti-FLAG M2 (Sigma-Aldrich) diluted 1:200 for 1 hour. Non-bound primary antibodies were washed in three changes of blocking buffer. Cells were stained for 1 hour with Alexa-488 conjugated goat anti-mouse antibody (Molecular Probes) diluted 1:300 in blocking buffer and washed with blocking buffer. Chromosomal DNA was stained by addition of 5 µM ToPro3 iodide (Molecular Probes) to the secondary antibody solution.

Western blot analysis demonstrated that the expression levels of HIV-1 IN in the cells transfected with 76A siRNA were reduced dramatically (not shown), suggesting that Inip76 is necessary for stability of the IN protein in human cells, likely protecting IN from proteolysis.

Example 11. Recombinant Inip76-IN Complex Produced in Escherichia coli.

As elaborated above, recombinant Inip76 is a potent stimulator of HIV IN activity *in vitro*. We wanted to reconstruct the Inip76-IN complex from recombinant proteins, as such complex can be potentially used to study interactions of the proteins on molecular level as well as an enzyme for DNA recombination *in vitro* and *in vivo*. Biochemical studies on HIV IN as well as other retroviral INs suffer from poor solubility of the proteins. We found that adding purified recombinant Inip76 to IN solutions had a solubilising effect on the IN protein. Thus, when both proteins were mixed at a concentration of 0.25 mg/ml in 150 mM NaCl/10 mM Hepes/1 mM $MgCl_2$/0.1 % NP40,

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Approximately 50 % of the IN protein remained soluble after 45 minutes incubation at 4 °C and centrifugation at 20000 g for 5 minutes. In contrast, only about 5 % of IN was recovered in soluble form when Inip76 was omitted from the mixture. This result indicates that the complex between IN and Inip76 is more soluble than free recombinant IN, and that such complex can be formed using recombinant proteins. A soluble form of retroviral integrase in complex with its chromosomal receptor might help to determine the structure of the active form of the retroviral IN. In addition, if active such complex could be used as an enzyme for *in vitro* DNA recombination.

i. Construction of the Inip76 Expression Plasmid pCP-Nat75

The open reading frame of Inip76/LEDGF was PCR amplified from pCP6H75 using the primers 5'-TGACTCGCGATTTCAAACC and 5'-CCGCGAATTCTAGTTATC TAGTGTAGAATCCTTC. The resulting DNA fragment was digested with *Eco*RI and subcloned between *Nde*I (treated with T4 DNA polymerase to obtain blunt terminus) and *Eco*RI sites of the pRSETB vector (Invitrogen). The complete Inip76 open reading frame and the phage T7 promoter region of the resulting plasmid, pCP-Nat75, was sequenced to confirm that no mutation occurred. The plasmid was transformed into the *E. coli* PC1 strain (E. coli BL21(DE3), *ΔendA*::Tc^R, pLysS) (Cherepanov et al., 1999) by standard methods. The resulting strain, PC2LEDGF expressed Inip76 upon induction with IPTG.

ii. Construction of the IN Expression Plasmids pKB-IN6H and pKB-A15IN6H

To construct the plasmid for bacterial expression of HIV-1 IN with C-terminal His_6 tag, we amplified the full IN open reading frame using PCR with the primers 5'-GCGCG TCGACATCCTCATCCTGTCTAC and 5'-AATACGACTCACTATAGGG from the pINSD plasmid (obtained from The NIH AIDS Research and Reference Reagent Program, catalog #2820). The RCR fragment was digested with *Nde*I and *Sal*I and subcloned between *Nde*I and *Sal*I sites of pET-20b(+) (Novagen). The open reading frame of IN as well as the T7 promoter region of the resulting plasmid pKB-IN6H was sequenced to verify absence of mutations. When the plasmid was transformed into PC1 bacteria, it expressed HIV-1 C-terminally His_6-tagged IN. However, pKB-IN6H is not able to stably co-exist with pCP-Nat75, as both plasmids have the same type of

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replication origin. To obtain pKB-A16IN6H, an IN-expression plasmid *compatible* with pCP-Nat75, we inserted the *BglII/Dra*III fragment of pKB-IN6H between *Bam*HI and *Bsa*I sites of pACYC177 vector (Chang and Cohen, 1978) (available from New England Biolabs) (the *Dra*III and *Bsa*I ends of the DNA fragments were treated with T4 DNA polymerase to obtain ligatable blunt termini).

iii. Co-expression of Inip76 and His_6-tagged HIV-1 IN in *Escherichia coli*

The plasmid pKB-A15IN6H was transformed into the PC2LEDGF *E. coli* strain (BL21(DE3), *ΔendA*::Tc^R, pLysS, pCP-Nat75) by standard methods. After transformation, bacteria harboring the newly transformed plasmids were selected on LB agar plates containing 120 µg/ml ampicillin, 40 µg/ml kanamicin and 15 µg/ml tetracycline at 28 °C. This procedure resulted in introduction of the pKB-A15IN6H plasmid and knock-out of the incompatible pLysS plasmid. The resulting strain PC2LEDGF-C (BL21(DE3), *ΔendA*::Tc^R pCP-Nat75, pKB-A15IN6H) was grown on LB agar plates and in liquid LB medium supplemented with 120 µg/ml ampicillin, 40 µg/ml kanamicin and 15 µg/ml tetracycline at 28 °C. Expression of Inip76 and IN was induced by addition of 1 mM IPTG at 29 °C in LB medium.

iv Purification of the Inip76-IN Complex Expressed in *E. coli*

An overnight culture of PC2LEDGF-C (100 ml) was diluted with 4 l of fresh LB medium supplemented with 120 µg/ml ampicillin and 40 µg/ml kanamicin and grown in a shaking incubator at 28 °C until its optical density (measured at 600 nm) reached approximately 0.8, then the culture was induced with 1 mM IPTG. After 3 hours expression at 29 °C the bacteria were collected by centrifugation and kept frozen overnight at –70 °C. Next day defrozen bacteria were re-suspended in 20 ml of cell breaking buffer (500 mM NaCl/30 mM Hepes/1 mM $MgCl_2$/0.1 mM PMSF) and lysed by two passages through a French press at a cell pressure of 18000 psi. The resulting bacterial lysate was cleared by centrifugation at 15000 g for 30 min supplemented with 25 mM imidazol and mixed with 1.5 ml of Ni-NTA resin (Qiagen) equilibrated with the same buffer. The slurry was gently mixed for 30 minutes to allow His-tagged protein to

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bind to the resin. The resin was then packed into a disposable gravity flow column and washed with 50 ml of 400 mM NaCl/30 mM Hepes/1 mM $MgCl_2$/25 mM imidazol, pH 7.2 followed by 12 ml of 400 mM NaCl/30 mM Hepes/1 mM $MgCl_2$/35 mM imidazol, pH 7.2. The bound protein was then eluted in the elution buffer (400 mM NaCl/30 mM Hepes/1 mM $MgCl_2$/200 mM imidazol, pH 7.2). Both IN and Inip76 eluted in the presence of 200 mM imidazol, as can be seen from the SDS-PAGE gel shown in FIG. 9A. In a control experiment, carried-out with the lysate of induced PC2LEDGF, Inip76 was detected only in the non-bound fraction, and no Inip76 was detected in the fractions eluted with 200 mM imidazol (FIG. 9B). Therefore, as was expected, in the absence of His_6-tagged IN, Inip76 does not bind to Ni-NTA confirming that all Inip76 found in the samples is indeed associated with IN.

The fractions containing IN and Inip76 were pooled together and injected into a 1 ml HiTrap SP Sepharose column (Amersham-Pharmacia) equilibrated with 35 % chromatography buffer B in A (buffer A was 1 mM $MgCl_2$/30 mM Hepes, pH 7.2, buffer B was 1 M NaCl/1 mM $MgCl_2$/30 mM Hepes, pH 7.2). The column was washed with 35 % B at 0.7 ml/min and the bound proteins were eluted in a 15 ml linear gradient from 35 % B to 90 % B. The host-derived contaminating proteins, including the 70 kDa band, as well as free IN protein were found in the flow-through fraction (FIG. 9C). The Inip76-IN complex eluted in approximately 55 % B, corresponding to 550 mM NaCl (fractions 16-22 in FIG. 9C). The fractions containing Inip76 and IN were pooled and concentrated by ultrafiltration using Centricon-30 (Millipore) to a final volume of approximately 70 µl (FIG. 10A). The final protein concentration was determined to be 4.8 mg/ml using BCA kit (Pierce) and BioRad protein assay (BioRad) with bovine serum albumin as a standard. The protein was kept at 4 °C for a week without signs of aggregation. In contrast, free HIV IN was not soluble at these concentrations in buffers with physiological pH. Detergents such as CHAPS or NP40 were needed to keep the protein from aggregation even at protein concentrations below 1 mg/ml.

Results

Example 12: HIV-1 IN is present in the insoluble nuclear fraction

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The 293T-IN^{s}ala cell line used in this work was similar to the previously reported 293T-IN^s, except that it expresses HIV-1 IN with the Met-Ala dipeptide at its N-terminus (instead of Met-Gly) (Cherepanov et al., 2000). This change was introduced to prevent potential myristoylation of the protein (Boutin, 1997) and affected neither cell line stability nor IN expression levels. The integrase protein was nuclear in both cell lines as determined by indirect immunofluorescence microscopy (data not shown and Cherepanov et al., 2000). After centrifugation of the digitonin- or NP40-treated 293T-IN^{s}ala cells, most of the IN protein was retained in the nuclear pellet (Figure 1A). Since NP40 permeabilizes both the plasma membrane and the nuclear envelope, the bulk of IN present in the cell is thus stably associated with insoluble nuclear structures. IN was extracted from the nuclei by increased salt concentrations; 350–500 mM NaCl was sufficient to elute the protein (Figure 1B). Therefore, buffers supplemented with 400 mM NaCl were used for preparing nuclear salt extracts.

Some chromatin-associated proteins can be eluted from detergent-permeabilized nuclei by gentle treatment with DNAse I or micrococcal nuclease (Fujita et al., 1997; Holthoff et al., 1998; Meller and Fisher, 1995). We observed no elution of IN when nuclei prepared from 293T-IN^{s}ala cells were treated with DNase I (Figure 1C), that completely digested nuclear DNA to fragments of less then 200 bp (data not shown). In accordance with Fujita et al. (Fujita et al., 1997), MCM3, a chromosomal replication factor, was removed from the nuclei by gentle DNase I treatment (Figure 1C). The same salt concentration was required to extract IN from the nuclease-digested nuclei (data not shown). However, subjection of the DNase-digested nuclei to low ionic strength conditions led to efficient elution of the protein (Figure 1D). Non-digested nuclei did not release any detectable amount of IN in the hypotonic conditions (Figure 1D). These results indicate that IN is associated both with chromosomal DNA and with some other nuclear structure, which is destabilized in low ionic strength conditions. Similar results were obtained when 293T-IN^{s}ala nuclei were exposed to micrococcal nuclease (data not shown).

Example 13: Chemical Cross-linking of IN Complexes Present in Nuclear Extracts

We used 3,3'-Dithiobis[sulfosuccinimidyl propionate] (DTSSP), an amine-specific N-hydroxysuccinimid ester with a 12 Å spacer arm, to cross-link protein complexes present in the nuclear extracts of 293T-IN^{s}ala cells. The nuclear proteins were extracted from the

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NP40-treated 293T-IN^{c}ala nuclei using the cytoskeleton buffer containing 400 mM NaCl (400CSK). The total protein concentration was adjusted to 100, 20 and 4 µg/ml; the samples were incubated with DTSSP and separated in a non-reducing 4-12% SDS PAGE gel. IN-containing cross-linking adducts were detected by western blotting using polyclonal anti-IN antibodies. A typical result is shown in Figure 2A. In the non-cross-linked samples (lanes 2, 6 and 10) as well as in the samples cross-linked in the presence of SDS (lane 1) only IN monomer and a band corresponding to IN dimer (approximately 60 kDa) were apparent. Addition of 0.1-2 mM DTSSP yielded cross-linked complexes of 60 kDa (p60^{cl}), 150-180 kDa (the p150^{cl} band, clearly visible in lanes 7, 8 and 12), 250-350 kDa (p300^{cl}, lanes 7 and 8) and less resolved higher molecular weight (MW) species. Strikingly, detection of the cross-linked IN complexes with our polyclonal anti-IN antibodies was far more sensitive than detection of the non-cross-linked IN (compare the lanes 2 and 4, for example). Probably, some strong conformational epitopes are better preserved within cross-linked IN during SDS PAGE and western blotting. No unspecific bands were revealed in nuclear extracts from 293T cells before and after cross-linking with DTSSP confirming that all bands detected in the western blot correspond to IN-containing complexes (data not shown). The cross-linking of the 293T-IN^{c}ala nuclear extract was clearly dependent on the concentration of both DTSSP and protein. Saturating cross-linking of diluted protein samples followed by denaturing SDS PAGE has been used to determine MWs of native protein complexes (see (Corey et al., 1998) and references therein). Cross-linking of the diluted nuclear extract (4 µg/ml protein) with 2 mM DTSSP yielded a band of approximately 150 kDa (p150^{cl}, lane 16), while in more concentrated extracts (100 µg/ml) a diffuse band of 250-300 kDa was the most prominent (p300^{cl}, lane 8). Thus, there exist at least two different IN complexes: a large complex at higher protein concentrations and a smaller complex in the diluted extract. The p150^{cl} product seems to be the result of complete cross-linking since no significant change in cross-linking occurs when the concentration of DTSSP was increased from 0.5 to 2 mM (compare lanes 15 and 16) and higher (not shown). Thus, p150^{cl} probably represents the IN complex present in the diluted nuclear extract. Moreover, this complex is a dissociation product and importantly, a component of the larger complex, since it appeared as a partial cross-linking adduct in the reactions with more concentrated protein extracts at 0.5 mM DTSSP and it decreased at 2 mM DTSSP (compare lanes 7 and 8). However, the p300^{cl} band is probably not the result of complete cross-linking of the larger

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complex, since a strong smear and some less resolved bands are present above $p300^{cl}$ on the western blot; aggregated material not able to enter 4% acrylamide gel is also evident (lanes 8 and 12). Some of the high MW adducts in the reactions with 100 µg/ml extracts may result from non-specific inter-molecular cross-linking of proteins. Cross-linking of IN complexes in the nuclear extracts using oxidizing Cu^{2+}-[1,10-phenanthroline]$_3$ complex (Cys-Cys cross-linker (Ji, 1983)) were also suggestive for the presence of a large protein complex that dissociated upon dilution releasing a molecule of approximately 150 kDa (data not shown).

Example 14: Apparent Stokes Radii of the two Nuclear Integrase Complexes

To confirm the presence of both IN complexes and deduce their size we used gel filtration. Undiluted (550 µg/ml protein) and diluted (30 µg/ml) nuclear salt extracts from 293T-IN^{e}ala cells were run on a calibrated Superdex 200 column and the IN elution was followed by immunoblotting the collected fractions (Figure 3A). We observed two distinct elution volumes corresponding to two different IN complexes. After chromatography of the undiluted extract, IN eluted symmetrically with a peak maximum in fractions 8 and 9 (elution volume, $V_e = 11.2$ ml; Stokes radius, $R_s = 61$ Å, see Figure 3B). However, IN eluted later and in a broader peak with a V_e of 14.1 ml ($R_s = 41$ Å, Figures 3A and 3B) when the sample was diluted prior to gel filtration. Assuming both complexes are globular, their MWs can be calculated to be 380 kDa and 115 kDa respectively (Figure 3C). The smaller dilution-resistant molecule ($R_s = 41$ Å) most likely corresponds to the $p150^{cl}$ cross-linked complex observed in the previous experiment, while partial cross-linking of the 61 Å IN complex probably resulted in $p300^{cl}$. The effective dilution of the peak fractions on the column was 5- to 7- fold as calculated from calibration runs (data not shown). Thus, the sample concentrations used in our gel filtration experiments corresponded to those used in the cross-linking experiments (100 µg/ml and 4 µg/ml).

Example 15: Purification and Characterization of FLAG-Tagged IN Complexes

To facilitate isolation of native IN complexes from cell extracts we modified the IN expression construct adding the FLAG epitope tag at the carboxy terminus of IN. The 293T-IN^{e}alaFLAG cell line, obtained by stable transfection of 293T cells with the tagged expression construct, was very similar to 293T-IN^{e}ala in stability and levels of IN

expression (not shown). FLAG-tagged IN (IN_f) localized predominantly in the nuclei in a diffuse pattern and was associated with chromosomes during mitosis (see below) as has been previously reported for non-tagged HIV-1 IN (Cherepanov et al., 2000). IN_f could be extracted from the nuclei of 293T-IN^{c}alaFLAG cells in the same conditions as for non-tagged IN. The cross-linking pattern of IN_f with DTSSP was very similar to that of non-tagged IN (Figure 2B). The two major cross-linking products of IN_f showed slightly slower migration in SDS PAGE gels than the original p150^{cl} and p300^{cl}, which can be attributed to the negative charge of the FLAG tag and increased molecular weight of the tagged protein. For convenience, however, we refer to the IN_f cross-linking adducts as p150^{cl} and p300^{cl}. The gel filtration profiles were as observed for the non-tagged IN extracted from 293T-IN^{c}ala cells (see below and data not shown).

In initial immunoprecipitation experiments, we incubated diluted nuclear extracts from metabolically labeled 293T-IN^{c}alaFLAG cells with the anti-FLAG M2 antibody and protein G agarose overnight. The protein isolated in this way displayed a single specific band in SDS PAGE gels migrating at the expected position for the FLAG-tagged IN (33.5 kDa) (Figure 4A). Isoelectrofocusing of immunoprecipitated IN_f in denaturing pH gradients showed a major band close to the predicted pI (6.5), which reacted with anti-IN serum in immunoblot (data not shown). When the IN_f immunoprecipitated from a nuclear extract of 293T-IN^{c}alaFLAG cells was eluted from the anti-FLAG M2 antibody with synthetic FLAG peptide and incubated with DTSSP, the p150^{cl} cross-linking product was readily obtained (Figure 4B). When higher IN_f concentrations were used in cross-linking, the immunoreactive reaction products accumulated at the top of the gel, suggesting aggregation of the protein (data not shown). We were not able to find reaction conditions to reproduce the p300^{cl} cross-linking product with IN_f preparations purified this way. Fractionation of purified IN_f on a Superdex column showed a peak with a K_{av} value very close to that of the 41 Å complex (Figure 4C). The fact that the 41 Å complex exists in the purified IN_f preparation and the apparent MW (115-150 kDa), based on cross-linking and gel filtration experiments, suggest that the 41 Å molecule is a tetramer of IN. Apparently, the native 61 Å IN complex was not stable enough to withstand immunoprecipitation under these conditions. Next, we tried shorter immunoprecipitation times (3-5 hours) starting from more concentrated nuclear extracts. Although the overall yield of IN_f decreased, the immunoprecipitated samples were found to contain an additional protein. It had an apparent MW of approximately 76 kDa, as determined by

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SDS PAGE (Figure 5A) and was present at variable ratios to IN_F in different preps. This protein, here referred to as Inip76 (for 76 kDa IN interactor protein), is specifically associated with IN_F since, it could not be immunoprecipitated from the parental 293T cells with the anti-FLAG antibody (compare lanes 2 and 3 in Figure 5A). When undiluted nuclear extract from 293T-IN^{a}alaFLAG cells was immunoprecipitated with anti-FLAG antibody for 4 hours both IN_F and Inip76 bands were readily detected (Figure 5B). Although extending immunoprecipitation to 16 hours improved IN_F recovery, the yields of Inip76 were greatly reduced (compare the lanes 1 and 2 in Figure 5B). Intriguingly, the p300^{a} band, detected after DTSSP cross-linking of the nuclear salt extracts, was readily observed when the Inip76-containing IN_F preparations were cross-linked with DTSSP (Figure 6E), suggesting that Inip76 is part of a large IN complex present in the nuclear extracts.

Example 16: Identification of the Inip76 Protein as LEDGF/DFS70/p75

By upscaling immunoprecipitation we were able to isolate sufficient amounts of Inip76 for characterization by Edman degradation and mass spectrometry (Figure 6A). The amino terminal sequence obtained from Inip76 was XXRDFKPGD (the first two residues being not resolved due to background noise). Scanning the TrEMBL protein database for human proteins carrying this sequence tag (Wilkins et al., 1996) resulted in four hits with accession numbers O95368, Q9UER6, Q9NZI3, O75475, all corresponding to the two alternative products of one gene: LEDGF/DFS70/p75 (lens epithelium-derived growth factor, referred hereafter as LEDGF) and the p52 protein ((Ge et al., 1998; Singh et al., 2000)). Although the actual MW of LEDGF is approximately 60 kDa, it has been reported to migrate as a 75 kDa band in SDS PAGE gels (Ge et al., 1998). On-line liquid chromatography tandem mass spectrometry analysis (LC/MS/MS) of tryptic peptides obtained by in-gel digestion of Inip76 provided further evidence that Inip76 is indeed identical to LEDGF. Lens epithelium-derived growth factor (LEDGF), is a member of the epatoma-derived growth factor family, is found at low levels in many actively dividing and long lived cells. Its gene yields tow proteins, LEDGF/p75 and p52, by alternative splicing (Ge, H., Si, Y., and Roeder, R. G. (1998) EMBO J. 17, 6723-6729 and 2). LEDGF belongs to a family of homologous proteins including hepatoma-derived growth factor (HDGF) (Nakamura, H, Izumoto, Y, Kambe, H, et al (1994) Molecular cloning of complementary DNA for a novel human hepatoma-derived growth factor: its homology

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with high mobility group-1 protein J Biol Chem 269, 25143-25149) and HDGF-related protein-1 and -2. (Izumoto, Y, Kuroda, T, Harada, H, Kishimoto, T, Nakamura, H. (1997) Hepatoma-derived growth factor belongs to a gene family in mice showing significant homology in the amino terminus Biochem Biophys Res Commun 238,26-32).

Half of the predicted LEDGF tryptic peptides within the mass range of 1000-2500 Da could be identified in the sample and their MS/MS spectra readily matched LEDGF covering approximately 18% of its sequence (Figure 6B and Table1). Moreover, Inip76 strongly reacted with a commercially available monoclonal anti-LEDGF antibody (see below and data not shown). Most of IN_f present in nuclear extracts could be immunoprecipitated with the anti-LEDGF antibody, whereas only about 10 % of LEDGF could be recovered with the anti-FLAG antibody (Figure 6C), suggesting that LEDGF is present in an excess over IN_f in the extract. Non-tagged IN could also be efficiently precipitated with the anti-LEDGF antibody, and a fraction of the LEDGF could be precipitated with polyclonal anti-IN antibody in similar conditions from nuclear salt extracts prepared from 293T-IN^{s}ala cells.

Example 17: Inip76 is part of the 61 Å HIV IN complex

To determine whether the 61 Å complex contains Inip76/LEDGF, we pre-incubated the nuclear salt extract from 293T-IN^{s}alaFLAG cells with a monoclonal anti-LEDGF antibody prior to chromatography on a Superdex column. The IN_f elution profile changed dramatically: the peak eluted now near the void volume of the column (Figure 6D). Elution of the 61 Å complex was not altered by pre-incubation of the extract with an unrelated mouse IgG1 (Figure 6D). Predictably, elution of the 41 Å IN complex (the presumed IN tetramer) did not change after pre-incubation of the diluted nuclear extracts with the anti-LEDGF antibody.

When IN_f-Inip76 complex was purified by immunoprecipitation and cross-linked with DTSSP, the $p300^{cl}$ band could be readily detected in an immunoblot with anti-IN antibody (Figure 6E). However, $p300^{cl}$ did not react with a monoclonal anti-LEDGF antibody; instead a western blot with the anti-LEDGF antibody revealed two bands migrating at higher positions in the gel ($pHMW_1^{cl}$ and $pHMW_2^{cl}$, Figure 6E) (the MWs of these molecules are too high to be determined from SDS PAGE). These results suggest that $p300^{cl}$ is a product of incomplete cross-linking of the 61 Å IN-Inip76 complex. Being composed of IN alone, $p300^{cl}$ probably represents an octamer of IN (a dimer of

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tetramers). Contacts between Inip76 and IN within the 61 Å complex may be less prone to cross-linking with DTSSP than those between IN protomers. We cannot, however, exclude the possibility that the target epitope for the monoclonal anti-LEDGF antibody used is masked or destroyed within $p300^{cl}$. In addition to the major $p300^{cl}$ product, a band at a position close to $pHMW_1^{cl}$ is present on the anti-IN immunoblot of the purified and cross-linked IN_f-Inip76 complex (Figure 6E; lane 2). Thus, the $pHMW_1^{cl}$ adduct is probably the smallest cross-linked IN complex containing Inip76.

Example 18: Inip76 co-localizes with IN within nuclei of 293T-IN°alaFLAG cells

In mammalian cells, LEDGF is expressed as a nuclear protein; it is distributed in a diffuse manner throughout the nucleus during interphase and is stably associated with condensed chromosomes throughout metaphase and anaphase (Nishizawa et al., 2001). A similar subcellular distribution has been reported for HIV-1 IN (Cherepanov et al., 2000). Immunofluorescent detection of both IN_f and Inip76/LEDGF in fixed 293T-IN°ala cells revealed strikingly similar intranuclear distribution patterns for both proteins (Figure 7A). In accordance with previous reports, both proteins were bound to condensed chromosomes in mitotic cells (Figure 7B). The distribution of another nuclear protein, the catalytic subunit of DNA-dependent protein kinase (DNA PKcs) clearly differed from that of IN_f (Figure 7C). In addition, DNA PKcs was excluded from condensed chromosomes in mitotic cells (data not shown). Intriguingly, the nuclear localization of IN_f and Inip76 did not correspond to the overall DNA staining pattern, arguing against the possibility that the apparent co-localization of the two proteins might be merely due to their independent association with chromosomal DNA. In a control experiment, we visualized IN_f using a mixture of polyclonal and monoclonal anti-FLAG antibodies; the obtained two-color IN_f staining was similar to that of IN_f and Inip76 (data not shown).

Example 19: Inip76 is an activator of HIV-1 IN in vitro

We have previously described activities of recombinant HIV-1 IN on the mini-HIV substrate, a linear 4.7 kb double stranded DNA molecule, carrying the U3 and U5 terminal fragments of the viral LTR sequences (Cherepanov et al., 1999). Recombinant HIV-1 IN on itself was proficient in carrying-out cleavage and strand transfer using this long DNA substrate; however, presence of 5-12% polyethylene glycol (PEG) in the reaction was required for the enzymatic activity. To ascribe a possible function to the

observed Inip76-IN interaction, we examined whether Inip76 could modulate enzymatic activity of HIV-1 IN in vitro. The mini-HIV substrate was incubated with recombinant IN and His_6-tagged Inip76 in the absence of PEG, and the reaction products were analyzed by native agarose gel electrophoresis (Figure 6F). In the absence of Inip76, strand transfer products were almost undetectable (lane 3). Addition of Inip76 resulted in a potent stimulation of the reaction. In some conditions, approximately half of the substrate DNA was converted into various reaction products including those that were too large to enter the gel (lanes 7 and 8). Remarkably, both the overall efficiency of the reaction and the range of the strand transfer products depended on the concentration of Inip76. No significant variation in the yield of the strand transfer products was detected when the order of addition of Inip76 and IN to the mini-HIV reaction was reversed (data not shown). Although addition of Inip76 to IN reactions containing short oligonucleotide substrate stimulated the strand transfer activity, presence of PEG and DMSO was still essential for efficient Mg^{2+}-dependent activity of our recombinant enzyme preparation (Cherepanov et al., 1997)).

Example 20: Disruption of the nuclear and chromosomal localization of HIV IN using small interfering RNA molecules specific to the Inip76 mRNA.

Immunofluorescent detection of Inip76 and IN revealed that Inip76 expression was efficiently knocked-down in the majority of cells transfected with 76A, 76B and 76C molecules. Moreover, IN re-distributed from the nuclei to the cytoplasm of the transfected cells as illustrated in the FIG. 8B. Importantly, after knock-down of Inip76 expression, IN was not able to bind to chromosomal DNA, which was evident in all mitotic cells with condensed chromosomes (FIG. 8C). The distribution and expression of IN and Inip76 remained unchanged in mock-transfected cells or cells transfected with NC RNA (FIG. 8A).

Example 21: Recombinant Inip76-IN Complex Produced in Escherichia coli: Activity of the Purified Recombinant IN-Inip76 Complex

The purified complex was supplemented with the IN mini-HIV substrate in the conditions similar to the mini-HIV reactions described above. The final reactions

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contained 1.5, 0.75, 0.37, 0.18 and 0 μg of the IN-Inip76 complex, 150 ng of the mini-HIV substrate in the final volume of 20 μl. The reactions also contained 110 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 20 mM Hepes, pH 7.5. After 90 min incubation at 37 °C, the reactions mixtures were supplemented with 0.2 % SDS, 2 mM EDTA plus 5 μg of proteinase K and further incubated at 37 °C for 20 min, to disrupt all protein-DNA complexes. The reaction products separated in 0.8 % agarose gel were detected by staining with the SybrGold stain (Molecular Probes) (FIG. 10B). The activity of the complex was similar to the activity of IN in the presence of separately added Inip76 as shown above.

Discussion

The minimal nuclear HIV-1 IN complex is a homotetramer

A serious obstacle in working with recombinant retroviral integrases is their poor solubility and propensity for aggregation. When produced in bacteria, HIV IN has to be extracted from inclusion bodies; detergents are used to stabilize the enzyme in solution. All crystal structure and some in vitro multimerization studies have been done with the soluble mutants. Furthermore, recent reports raised concern that the stoichiometry and enzymatic activities of recombinant IN can be affected by the enzyme preparation (Leh et al., 2000; Sinha et al., 2002). Our goal was to study protein complexes that HIV-1 IN forms within nuclei of human cells. The bulk of HIV-1 IN present in 293T cells that stably produce this viral protein, is associated with the insoluble nuclear fraction. Although IN seems to be directly or indirectly bound to chromosomal DNA, this may not be the only factor in nuclear retention of IN, since digestion of the detergent-permeabilized nuclei with nucleases was not sufficient to elute IN. In this work, we concentrated on the study of IN complexes extracted from the detergent-permeabilized nuclei in hypertonic conditions. We found that salt-eluted IN exists as part of a distinct 61 Å complex, which is not stable in diluted nuclear extracts and dissociates, releasing a 41 Å core molecule. Our cross-linking and gel filtration data suggest that the latter molecule is a tetramer of IN. Indeed, the complex exists in immunoprecipitated IN_f preparations. Its molecular weight determined both by gel filtration and cross-linking (115-150 kDa) is close to the value expected for the presumed tetramer (128 kDa). Finally, partial cross-linking of the diluted nuclear extracts and of the purified tagged IN protein produced bands at the positions corresponding to the IN monomer, dimer and trimer, in addition to $p150^{cl}$ (data not shown and Figure 2A, lane 7). We estimate that the concentrations of IN and IN_f in the nuclear extracts did not exceed 10 nM ($\leq$ 0.3 µg/ml IN at 100 µg/ml total protein). Therefore, the IN tetramer was stable even at subnanomolar concentrations (i.e. in the extracts diluted to 4 µg/ml of total protein), implying that the minimal nuclear IN complex is a tetramer.

Composition of the 61 Å HIV-1 IN complex

The native 61 Å IN complex was found to decay during prolonged incubations and not to be stable during purification. When the immunoprecipitation time was shortened and

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higher initial extract concentrations were used, a cellular protein with an apparent MW of 76 kDa specifically co-precipitated with the epitope-tagged IN. The protein, or Inip76, as we refer to it, was found to be identical to human LEDGF/DFS70/p75 protein. We further confirmed that Inip76/LEDGF is a part of the native 61 Å IN complex, since addition of a monoclonal anti-LEDGF antibody shifted elution of the complex from a gel filtration column. Importantly, when LEDGF protein was present, the $p300^{cl}$ band became visible after DTSSP cross-linking of the immunoprecipitated IN_f. This band could not be reproduced in LEDGF-negative IN_f samples obtained following the long immunoprecipitation protocol. $P300^{cl}$ seems to be the result of partial cross-linking of the 61 Å IN complex for the following three reasons: (*i*) the MW was lower than the expected value for a 61 Å globular complex (≈400 kDa); (*ii*) DTSSP cross-linking products with apparent MWs higher than that of $p300^{cl}$ were visible on anti-IN immunoblots; (*iii*) this product did not react with anti-LEDGF antibody. Based on its apparent molecular weight, we speculate that $p300^{cl}$ probably represents two cross-linked IN tetramers.

What is the exact stoichiometry of the IN-Inip76 complex? At the concentrations it is present in nuclear extracts, the 61 Å complex was not stable enough to allow measurement of its sedimentation coefficient, which is required to determine its precise MW (Siegel and Monty, 1965). Assuming that the 61 Å complex is globular, we estimate its MW to be around 400 kDa. The simplest model compatible with this MW is of a symmetrical complex containing a pair of IN tetramers and two subunits of Inip76, which corresponds to a macromolecule of 370 kDa. Reconstitution of the IN-Inip76 complex from the recombinant proteins will help to confirm the proposed stoichiometry. At this time, we cannot rule out the possibility that the native 61 Å complex contains another cellular protein lost during immunoprecipitation. The purified Inip76-containing IN samples displayed complex gel filtration profiles, probably due to partial dissociation of the native complex.

Retroviral IN within PIC: a dimer of dimers or a dimer of tetramers?

During reverse transcription, the two retroviral cDNA termini are not completed simultaneously and both seem to be substrates for the 3'-processing activity of IN as soon as they appear (Miller et al., 1997). Moreover, at least in case of HIV, 3'-processing of one LTR end was observed when the second end was non-functional and also did not support normal intasome assembly (Chen and Engelman, 2001). On the other hand, two

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functional LTRs were found to be required for strand transfer activity of isolated HIV PICs. Therefore, although LTRs can be processed asymmetrically, a synaptic complex involving both LTRs must form to allow strand transfer, ensuring that only legitimate integration of both retroviral cDNA ends occurs. Based on a comparison to Mu phage transposase and available crystal structure data, it has been suggested that the active form of retroviral IN is a tetramer (a dimer of dimers) (Wang et al., 2001). Our results suggest that HIV IN expressed in human cells is indeed present as a stable tetramer. Recombinant HIV-1 IN as well has been recently reported to form stable tetramers in diluted solutions (Leh et al., 2000). Intriguingly, both Mu phage and Tn5 transposases form active multimers (tetramers and dimer respectively) only within their synaptic complexes. Thus, independent transposase subunits must first bind to the ends of the transposon genome, before being brought together to form the synaptic complex. Extrapolating this scheme to the retroviral PIC assembly, the stable IN tetramers can be looked at as such independent subunits, which first have to bind to one LTR end each before interacting with each other. According to this model, each individual tetramer would be capable of currying out 3'-processing reaction, whereas a dimer of tetramers would be necessary to accomplish the strand transfer. The data presented here suggest that the cellular HIV-1 IN 61 Å complex contains two IN tetramers that are close enough to be cross-linked to each other. It is therefore very tempting to speculate that this dimer of tetramers may represent the configuration of IN required for concerted integration.

What is the role of Inip76 in retroviral replication?

The human Ini1/hSNF5/BAF47 protein has been shown to interact with HIV-1 IN in a yeast two hybrid screen and in vitro. Neither did we observe co-immunoprecipitation of Ini1 with IN from nuclear extracts; nor were we able to detect co-localization of the two proteins in the cell (to be published elsewhere). We speculate that the cellular IN-Ini1 interaction might be transient and only occurring during certain steps of viral replication. We have now shown that IN complexes extracted from the cell lines stably expressing HIV-1 IN contained another human protein, Inip76, identical to LEDGF/DFS70/p75. IN_F and Inip76 co-localized in the nuclei of 293T-IN^FalaFLAG cells, confirming that the observed interaction was not an artifact of the extraction. So far, there has been no previous account of a link between LEDGF/DFS70/p75 and retroviral replication. No obvious relation seems to exist between this protein and known chromatin remodeling

factors. The protein has first been described as the positive transcription cofactor PC4-interacting protein (Ge et al., 1998). Two alternatively spliced cDNA clones were isolated from a HeLa library coding for two proteins p52 (333 amino acids, as predicted from the cDNA) and p75 (530 aminoacids) sharing identical 325 amino terminal residues. The transcripts coding for p75 and p52 were detected in different cell types and tissues, p52 being most abundant in testis and p75 in thymus. Independently, a cDNA clone coding for a protein identical to p75 has been isolated from a lens epithelium cell library (Singh et al., 1999). Overexpression of the protein stimulated survival of diverse primary cells and cell lines and enhanced their resistance to oxidative and hyperthermic stress (hence LEDGF, "lens epithelium derived growth factor"). The same protein has also been identified as the DFS70 autoantigen, antibodies to which were found in some cases of atopic dermatitis, asthma and interstitial cystitis (Ochs et al., 2000). One case of acute myeloid leukemia with a chromosomal translocation resulting in a fusion of the NUP98 and LEDGF genes has been described (Ahuja et al., 2000).

Based on sequence similarity, Inip76/LEDGF/DFS70/p75 is a member of the hepatoma-derived growth factor (HDGF) family that includes HDGF and several other HDGF-related proteins (HRPs) (reviewed in (Dietz et al., 2002)). High degree of homology exists between the amino terminal regions of these proteins. The PWWP motif (70 residues containing the Pro-Trp-Trp-Pro core sequence) is located within the amino terminal homology region of HRPs and relates them to a larger and functionally diverse nuclear protein family, that includes DNA-binding transcription factors and enzymes involved in DNA repair and DNA methylation. PWWP domains are thought to be implicated in protein-protein interactions (Stec et al., 2000).

An eye-catching feature of Inip76 is the abundance of the charged amino acids comprising approximately 40% of the total residues. Screening its sequence against the BLOKS+ protein motif database (Henikoff et al., 1999) revealed fragments with similarity to the HMG-I(Y) DNA AT hook sequence (data not shown). LEDGF has been shown to be a DNA-binding protein with affinity for heat shock and stress-related DNA elements (HSE and STREs) (Singh et al., 2001). It has also been reported to interact with components of the general transcription machinery and with the transcription activation domain of VP16 (Ge et al., 1998). Recent reports suggested that LEDGF plays an important role in regulating expression of the stress-response genes (Fatma et al., 2001; Shinohara et al., 2002). As mentioned above, alternative splicing of LEDGF pre-mRNA,

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allows expression of the second protein, p52, from the same gene. A growing body of evidence suggests that p75 and p52 may have different functions. Although they both can interact with PC4, VP16 and general transcription factors, at least in vitro, p52 displays higher transcription activation activity (Ge et al., 1998). In addition, p52 and not p75 has been shown to functionally interact with the ASF/SF2 splicing factor in vitro (Ge et al., 1998). The proteins also differ in their nuclear distribution patterns (Nishizawa et al., 2001). Intracellular levels of p52 appear to be much lower than those of p75, at least in the cell lines we have tested (HEK-293, 293T, HeLa, CEM) (data not shown). We have not detected co-immunoprecipitation of p52 with IN from the nuclear cell lysates of IN-expressing 293T cells. However, it remains to be determined whether p52 is able to interact with HIV IN.

What is the role of the Inip76-IN interaction in the retroviral life cycle?

The fact that recombinant Inip76 was able to stimulate HIV-1 IN activity in vitro suggests a direct involvement of Inip76 in the integration process. As a chromosome-associated IN-binding protein, Inip76 may serve as a docking factor for the PIC. It might thus be functionally similar to the Mu phage transposition co-factor MuB that associating with the acceptor DNA makes it a preferred target for transposition. It is also conceivable that Inip76 might be a part of the retroviral PIC. The experiments are currently underway to establish whether Inip76 is an essential factor in HIV replication. If proved to be essential, Inip76 will constitute a novel target for anti-retroviral therapy directed against its interaction with IN. Alternatively, a therapeutic strategy based on modified Inip76 protein, designed to capture the viral IN in a catalytically quiescent complex, may be envisaged.

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Legends to the Figures

Figure 1. Extraction of IN from nuclei of 293T-IN^{s}ala cells.

(A) 293T-IN^{s}ala cells were lysed in 100CSK buffer in the presence of 4 μg/ml digitonin or 0.5% NP40 on ice for 10 minutes. The supernatant (S) and nuclear pellet (P) fractions were recovered and analyzed by western blotting with an anti-IN antibody. The first lane contains the total cell extract.

(B) 293T-IN^{s}ala cells were lysed in 100CSK buffer supplemented with 0.5% NP40 on ice for 10 minutes, and the extracted nuclei were re-suspended in CSK buffer containing 0 – 500 mM NaCl. After centrifugation, supernatants (S) and nuclear pellets (P) were analyzed by western blotting with an anti-IN antibody. The total nuclear protein was loaded in the first lane (nuc).

(C) NP40-permeabilized nuclei from the 293T-IN^{s}ala cells, prepared as above, were incubated in 100CSK buffer with (+DNase I) or without (-DNase I) DNAse I (250 units/ml) at 25 °C for 10 (10') or 30 (30') minutes. Following centrifugation, the supernatants (S) and pellets (P) were separated in an 11% SDS PAGE gel, the upper part of which was used for the immunoblot to detect MCM3 (91 kDa) and the lower part to detect IN (32 kDa). The total cytoplasmic and nuclear protein fractions were loaded in the first (cyt) and the second (nuc) lanes, respectively.

(D) NP40-permeabilized nuclei from 293T-IN^{s}ala cells were incubated in 100CSK buffer with or without DNase I (250 units/ml) for 10 minutes, pelleted by centrifugation and resuspended in ice-cold hypotonic buffer (2 mM EDTA, 2 mM Hepes [pH7.5]). After centrifugation, supernatants (S) and pellets (P) were analyzed by western blotting with anti-IN antibodies. The first lane (nuc) contains total nuclear protein.

Figure 2. Cross-linking of the IN and IN$_{f}$ complexes with DTSSP.

(A) The nuclear extract from 293T-IN^{s}ala cells was prepared in the 400CSK buffer, was cross-linked with DTSSP and separated in a non-reducing 4-12% SDS PAGE gel. The IN-containing cross-linking adducts were detected by western blotting with a polyclonal anti-IN antibody. Prior to cross-linking, the extract was adjusted to 100 μg/ml (lanes 1-5), 20 μg/ml (lanes 6-9) or 4 μg/ml (lanes 10-13) of total protein. The concentration of DTSSP was 0.1 mM (lanes 3, 7, 11), 0.5 mM (lanes 1, 4, 8, 12) or 2.0 mM (lanes 5, 9,

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13). No cross-linker was added to the samples in lanes 2, 6 and 10. The sample in lane 1 was cross-linked in the presence of 0.2% SDS.

(B) Cross-linking of the FLAG-tagged IN complexes was done in similar conditions as in (A). Only lanes containing samples cross-linked with 2 mM DTSSP are shown; the sample in lane 1 was cross-linked in the presence of 0.2% SDS. The $p300^{cl}$, $p150^{cl}$, $p60^{cl}$, the IN monomer bands and the positions of the MW markers are indicated. The ≈470 kDa mark corresponds to the band of the catalytic subunit of DNA PK (MW 469 kDa), which was detected in a separate lane with a monoclonal anti-DNA PKcs antibody.

Figure 3. Determination of sizes and molecular weights of the IN complexes.

(A) Chromatography of nuclear extracts from 293T-IN^{s}ala cells was carried out on a calibrated Superdex 200 column. Prior to chromatography, the extract was adjusted to 550 µg/ml or 30 µg/ml of total protein; the collected fractions (1-26) were tested for the presence of IN by western blotting. The elution volumes (V_e) and the respective partition coefficients (K_{av}) for the observed IN peaks are indicated.

(B, C) Determination of the Stokes radii and approximate molecular weights of the IN complexes from the experimental K_{av} values. The partition coefficients for the standard proteins were determined in the same conditions (thyr., thyroglobulin, K_{av} = 0.039; ferritin, K_{av} = 0.17; catalase, K_{av} = 0.26; aldolase, K_{av} = 0.28; BSA, bovine serum albumin, K_{av} = 0.39; chymotr., chymotrypsinogen A, K_{av} = 0.59).

Figure 4. Immunoprecipitation of the FLAG-tagged IN from diluted nuclear extracts.

(A) Nuclear extracts prepared from metabolically labeled 293T-IN^{s}ala and 293T-IN^{s}alaFLAG cells were diluted to 200 µg/ml of total protein and immunoprecipitated with the anti-FLAG M2 antibody and protein G agarose beads for 16 hours at 4 °C. The protein was eluted by boiling in SDS PAGE sample buffer and separated in a 4-20% SDS PAGE gel. Radioautograph of the gel is presented.

(B) FLAG-tagged IN was immunoprecipitated from a diluted nuclear extract of non-labeled 293T-IN^{s}alaFLAG cells overnight. The protein was eluted with FLAG peptide in 400CSK buffer and cross-linked with DTSSP. The reaction conditions are similar to those in Figure 2.

(C) The protein immunoprecipitated and eluted as in (B) was subjected to gel filtration on a Superdex 200 column. The fractions collected (1-26) were analyzed by western blotting with anti-IN antibodies. The R_s value corresponding to the observed peak was determined as in Figure 3B.

Figure 5. Co-immunoprecipitation of FLAG-tagged IN with Inip76 from non-diluted nuclear extracts.

(A) Nuclear extracts from 293T-IN^{a}alaFLAG and 293T cells (700 µg/ml of total protein) were incubated with anti-FLAG M2 antibody and protein G agarose beads for 4 hours. The beads were washed as described in materials and methods and bound proteins were eluted with FLAG peptide in 400CSK buffer. The eluted proteins were concentrated by precipitation with TCA, re-dissolved in SDS sample buffer, separated in 4-20% denaturing PAGE gels and visualized by silver staining. Lane 1: immunoprecipitate of a nuclear extract from 60×10^6 293T-IN^{a}alaFLAG cells. Lanes 2 and 3: immunoprecipitation was done in parallel with nuclear extracts from 293T and 293T-IN^{a}alaFLAG cells. The bands of IN$_{\xi}$, Inip76, the heavy and the light chains of the anti-FLAG M2 IgG1 antibody as well as aprotinin (protease inhibitor present in 400CSK buffer) are indicated. Two sets of molecular weight markers were used in both gels to determine the apparent molecular weight of Inip76. Positions of the molecular weight markers are shown.

(B) Nuclear extract from 20×10^6 293T-IN^{a}alaFLAG cells (≈700 µg/ml total protein) was immunoprecipitated for either 4.5 hours (left lane) or 18 hours (right lane). The protein was then eluted and analyzed as in (A).

Figure 6. Inip76 is identical to LEDGF/DFS70/p75, appears to be a component of the 61 Å complex and stimulates IN activity in vitro.

(A) The Coomassie blue-stained PVDF membrane used for amino-terminal microsequencing of Inip76. The IN$_{\xi}$-Inip76 complex was eluted from protein G agarose-immobilized anti-FLAG M2 antibody with FLAG peptide, separated in reducing 4-20% SDS PAGE gel and transferred onto the PVDF membrane (lane 1). The proteins left on the beads after incubation with FLAG peptide were eluted with SDS sample buffer (lane 2). The bands corresponding to Inip76, IN$_{\xi}$, the heavy (H) and light (L) chains of the anti-FLAG M2 antibody, aprotinin and the MW markers are indicated.

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(B) Deconvoluted and sequence-converted MS/MS spectrum of a doubly charged peptide ion (m/z 982.57) obtained from the in-gel tryptic digest of Inip76 corresponding to the LEDGF peptide N425-K442. The observed b- and y-dominant fragment ions are indicated (Biemann, 1990).

(C) Co-immunoprecipitation of LEDGF and FLAG-tagged IN from a nuclear extract of 293T-IN^{F}alaFLAG cells. Immunoprecipitation was carried with anti-FLAG (lanes 2 and 2'), anti-LEDGF (lanes 3 and 3') or no antibody (lanes 1 and 1'). After 4 hours incubation, protein G agarose beads with precipitated protein complexes were washed with three changes of 400CSK buffer and re-suspended in reducing SDS PAGE sample buffer, followed by western blotting to detect IN_F and Inip76/LEDGF (lanes 1'-3'). The lanes 1-3 contain the immunoprecipitation supernatants.

(D) Elution of the 61 Å IN complex from a gel filtration column is shifted after pre-incubation with anti-LEDGF antibody. A nuclear extract of 293T-IN^{F}alaFLAG cells was preincubated with 3 µg/ml anti-HA (control IgG1) or anti-LEDGF antibody and separated by chromatography on a Superdex 200 column. IN_F was detected in the fractions by western blotting. The void volume of the column was 8.3 ml, approximately corresponding to fraction 2.

(E) Cross-linking of the Inip76-IN_f complex with DTSSP. The Inip76/INf complex was immunoprecipitated from a nuclear extract prepared from 293T-IN^{F}alaFLAG cells with anti-FLAG M2 antibody and protein G agarose for 4.5 hours. The protein was eluted with FLAG peptide and incubated with 2 mM DTSSP in the presence (lanes 1 and 1') or absence (lanes 2 and 2') of 0.2% SDS. The cross-linked samples were then separated in a non-reducing 4-12% SDS PAGE gel and immunoblotted with polyclonal anti-IN (left blot) or monoclonal anti-LEDGF (right blot) antibodies. The positions of IN_f and Inip76 as well as the cross-linking adducts $p150^{cl}$, $p300^{cl}$, $pHMW_1^{cl}$ and $pHMW_2^{cl}$ are indicated. Anti-FLAG M2 IgG1 present in the sample is detected on the anti-LEDGF western blot.

(F) Recombinant Inip76 enhances HIV-1 IN strand transfer activity in vitro. Mini-HIV DNA was pre-incubated with HIV-1 IN for 7 minutes at room temperature. Next, 0-0.8 µM His_6-tagged Inip76 was added to the reactions that were further incubated at 37°C for 90 minutes. The concentrations of IN and Inip76 used in the reactions are indicated. The reactions contained 125 ng mini-HIV DNA, 110 mM NaCl, 20 mM Hepes [pH 7.5], 5 mM DTT and 5 µM $ZnCl_2$ in a final volume of 20 µl. The reactions were stopped by addition of 0.5% SDS and 25 mM EDTA and the samples were digested with

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0.25 mg/ml proteinase K at 37 °C for 30 minutes to completely disrupt protein-DNA complexes. DNA was then precipitated with ethanol, re-dissolved in Tris-EDTA and analyzed by electrophoresis in an 0.8% agarose gel. The positions of the DNA molecular weight markers (23.1, 9.4, 6.6, 4.4 and 2.3 kb) are indicated. The gel was stained with SybrGold (Molecular Probes).

Figure 7. Co-localization of FLAG-tagged IN and Inip76/LEDGF in 293T-IN^{c}alaFLAG cells.

(A) Confocal laser scanning micrographs of a fixed and permeabilized cell fluorescently stained with a combination of monoclonal anti-LEDGF plus Alexa-488 conjugated anti-mouse antibodies to detect Inip76 (green, Inip76) and rabbit polyclonal anti-FLAG plus Alexa-555 conjugated anti-rabbit antibodies (red, IN) to localize FLAG-tagged IN. DNA was stained with To-Pro3 iodide (shown as white). The two-color merged image (IN+Inip76) was produced by overlaying the IN and Inip76 images.

(B) Both IN_f and Inip76 are associated with condensed chromosomes during mitosis. Immunofluorescent staining was performed as described in (A).

(C) DNA PKcs and IN_f display no significant co-localization. IN_f (red, IN) was detected as in (A); DNA PKcs (red, PKcs) was localized with monoclonal anti-DNA PKcs antibody plus Alexa-555 conjugated anti-mouse antibody. The two color IN+PKcs image is an overlay of the IN and PKcs images.

FIG. 8. Effect of RNA interference-mediated knock-down of Inip76/LEDGF expression in 293T-IN^{c}alaFLAG cells on HIV IN distribution.

Inip76 (A, B) and IN (C) were detected by indirect immunofluorescence in non-transfected cells (A), cells transfected with 76A siRNA (B, C). Immunofluorescence, chromosomal DNA staining and detection was done as described in Example VI.

FIG. 9. Purification of the Inip76-IN complex from bacteria co-expressing the two proteins.

(A) Elution of the IN-Inip76 complex from NiNTA Sepharose; 7 fractions collected were analyzed by 11% SDS PAGE and Coomassie staining. (B) A lysate of induced PC2LEDGF cells lacking His_6-tagged IN was incubated with NiNTA Sepharose and the

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bound proteins were eluted with 200 mM imidazol. The band corresponding to Inip76 is not present on this gel, confirming that free Inip76 is not associating with NiNTA Sepharose. (C) Further purification of the IN-Inip76 complex by cation exchange chromatography. The fractions 3, 4, 5 and 6 eluted from NiNTA (see panel A). They were pooled and loaded onto a 1 ml HiTrap SP Sepharose column. The complex was eluted with linear gradient of NaCl concentration. The non-bound (flow-through) fraction as well as the fractions #10 through #28 were analyzed by 11 % SDS PAGE and Coomassie staining. Positions of the molecular weight markers (kDa) are indicated.

FIG. 10. Concentration of the enzymatically-active IN-Inip76 complex.

(A) The complex purified by chromatography on NiNTA and SP Sepharose was concentrated using Centricon-30. Equal volumes of the original non-concentrated sample (lane 1), the intermediate samples (lanes 2, 3) and the final concentrated sample (lane 4) were separated by 11 % SDS PAGE. (B) Activity of the purified and concentrated recombinant IN-Inip76 complex was tested in the mini-HIV reaction in conditions described in the Example VII. Positions of the DNA molecular weight markers (kb) separated in a separate lane of the gel are indicated.

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Integrase Tetramers

Claims

1.A molecule which comprises a region specifically binding to a protein of the hepatoma-derived growth factor family or nucleic acids encoding said protein of the hepatoma-derived growth factor family, and suppresses or prevents retroviral replication.

2. The molecule of claim 1, characterised in that it prevents or suppress the protein of the hepatoma-derived growth factor family of interacting with retroviral integrase.

3. The molecule of claim 1 or 2, which comprises a region specifically binding to the protein Inip76 or nucleic acids encoding said Inip76, and suppresses or prevents viral replication.

4. The molecule of claim 3, characterised in that it prevents or suppress protein Inip76 of interacting with retroviral integrase.

5. The molecule of any the claims 1 to 4, characterised in that it suppresses or prevents retroviral replication.

6. The molecule of any the claims 1 to 4, characterised that it suppresses or prevents HIV replication.

7. A molecule according to any of the claims 1 to 6, which is chosen from the group

comprising an antibody or any fragment thereof; a small molecule specifically binding to Inip76; or nucleic acids encoding said Inip76; a ribozyme against nucleic acids encoding Inip76; and anti-sense nucleic acids hybridising with nucleic acids encoding Inip76.

8. Use of molecule according to any of claims 1 to 7 for the preparation of a medicament to treat or prevent retroviral infection.

9. Use of a molecule according to any of claims 1 to 7 for the preparation of a medicament to treat or prevent HIV infection.

10.A method to identify molecules according to claims 3 or 4, comprising exposing Inip76 or nucleic acids encoding said Inip76 to at least one molecule whose ability to suppress of Inip76 of interacting with a retroviral integrase protein is sought to be determined, determining binding or hybridising of said molecule(s) to Inip76 or to binding places on said integrase of Inip76 protein or to nucleic acids encoding said Inip76 monitoring the prevention or suppressing of retroviral replication or integration by the usage of at least one of said molecules.

12.A method to identify molecules according to claims 3 or 4, comprising exposing Inip76 or nucleic acids encoding said Inip76 to at least one molecule whose ability to suppress of Inip76 of acting as a cellular cofactor of retroviral integration is sought to be determined, determining binding or hybridising of said molecule(s) to Inip76 or to binding places on said retroviral integrase of Inip76 or to nucleic acids encoding said Inip76 monitoring the prevention or suppressing of retroviral integration by the usage of at least one of said molecules.

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13.A method to identify molecules according to claims 3 or 4, comprising exposing Inip76 or nucleic acids encoding said Inip76 to at least one molecule whose ability to suppress of Inip76 of promoting strand transfer activity of HIV is sought to be determined, determining binding or hybridising of said molecule(s) to Inip76 or to binding places on said integrase protein of Inip76 or to nucleic acids encoding said Inip76 monitoring the prevention or suppressing of HIV integration by the usage of at least one of said molecules.

14.A method for the production of a pharmaceutical composition comprising the usage of the method according to claims 9-12 and further more mixing said molecule identified, or a derivative or homologue thereof, with a pharmaceutically acceptable carrier.

15. A protein complex comprising a retroviral integrase and Inip76.

16. A protein complex comprising a recombinant retroviral integrase and a recombinant Inip76.

17. A method of stimulating retroviral integration using Inip76 or functional analogues thereof.

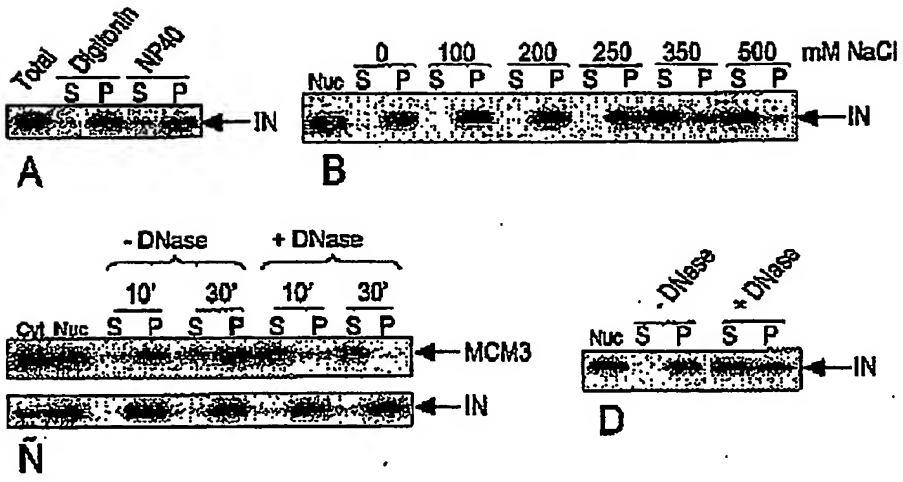
18. The use of Inip76 as a cellular cofactor of retroviral integration.

Integrase tetramers

Abstract

In a study of HIV-1 integrase (IN) complexes derived from nuclei of human cells stably expressing the viral protein from a synthetic gene it was demonstrated that in the nuclear extracts IN exists as part of a large distinct complex with apparent Stokes radius of 61 Å, which dissociates upon dilution yielding a core molecule of 41 Å. The IN complexes were isolated from cells expressing FLAG-tagged IN. By present invention it was demonstrated that the 41 Å core is a tetramer of IN, whereas 61 Å molecules are composed of IN tetramers associated with a cellular protein with an apparent molecular weight of 76 kDa. This integrase interacting protein (Inip76) was found to be identical to LEDGF/DFS70/p75, a protein implicated in regulation of gene expression and cellular stress-response. HIV-1 IN and Inip76 co-localized in the nuclei of human cells stably expressing IN. Furthermore, it has been demonstrated by present invention that recombinant Inip76 strongly promoted strand-transfer activity of HIV-1 IN in vitro. Our findings reveal that the minimal IN molecule in human cells is a tetramer and clearly demonstrates that Inip76 is likely to play a role in retroviral integration.

FIG. 1



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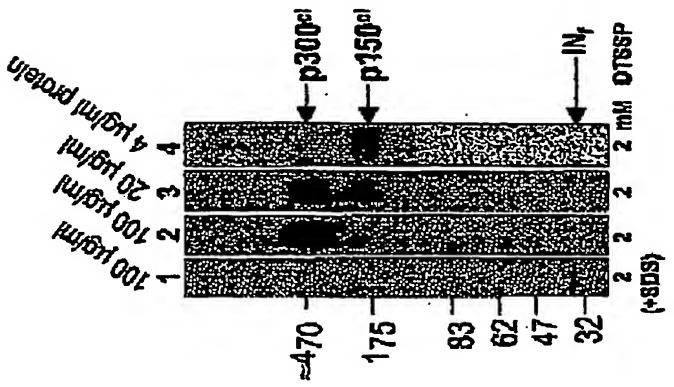

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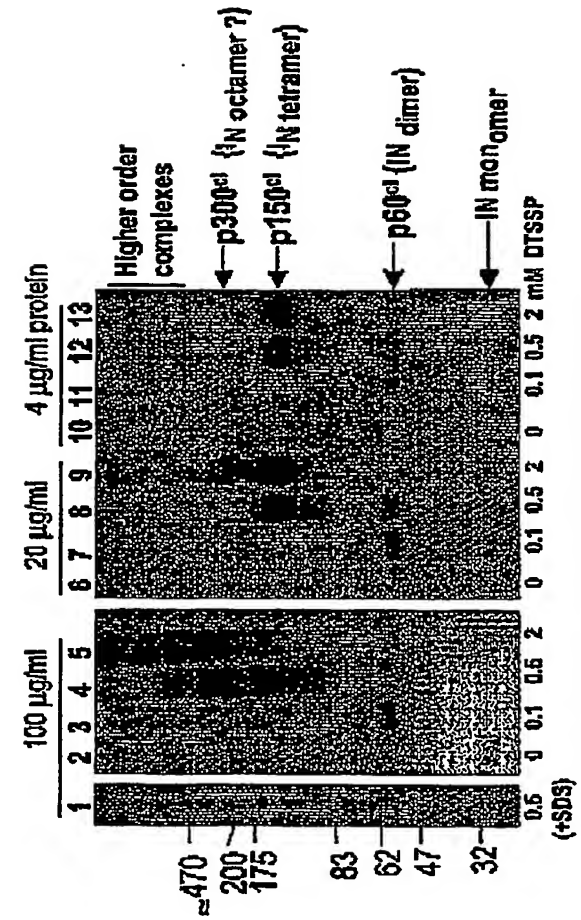

A

FIG. 2

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FIG. 3

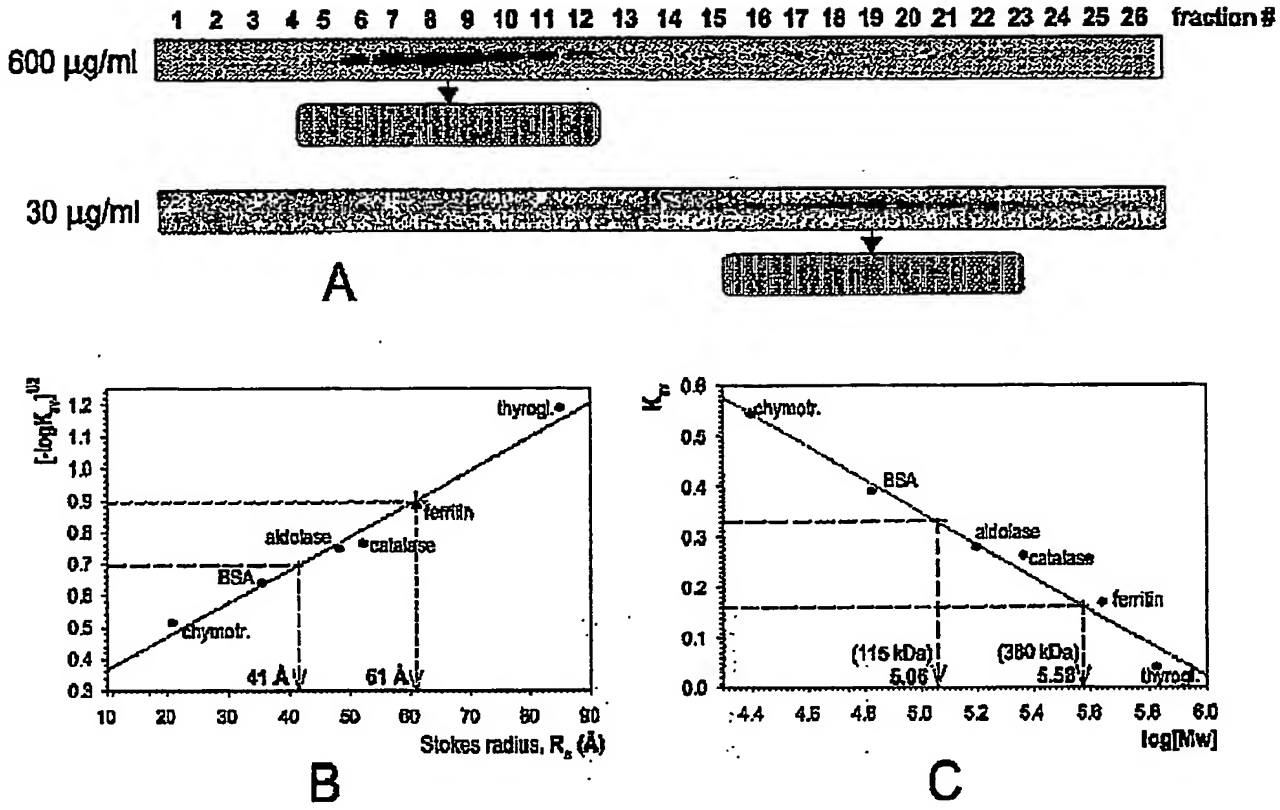

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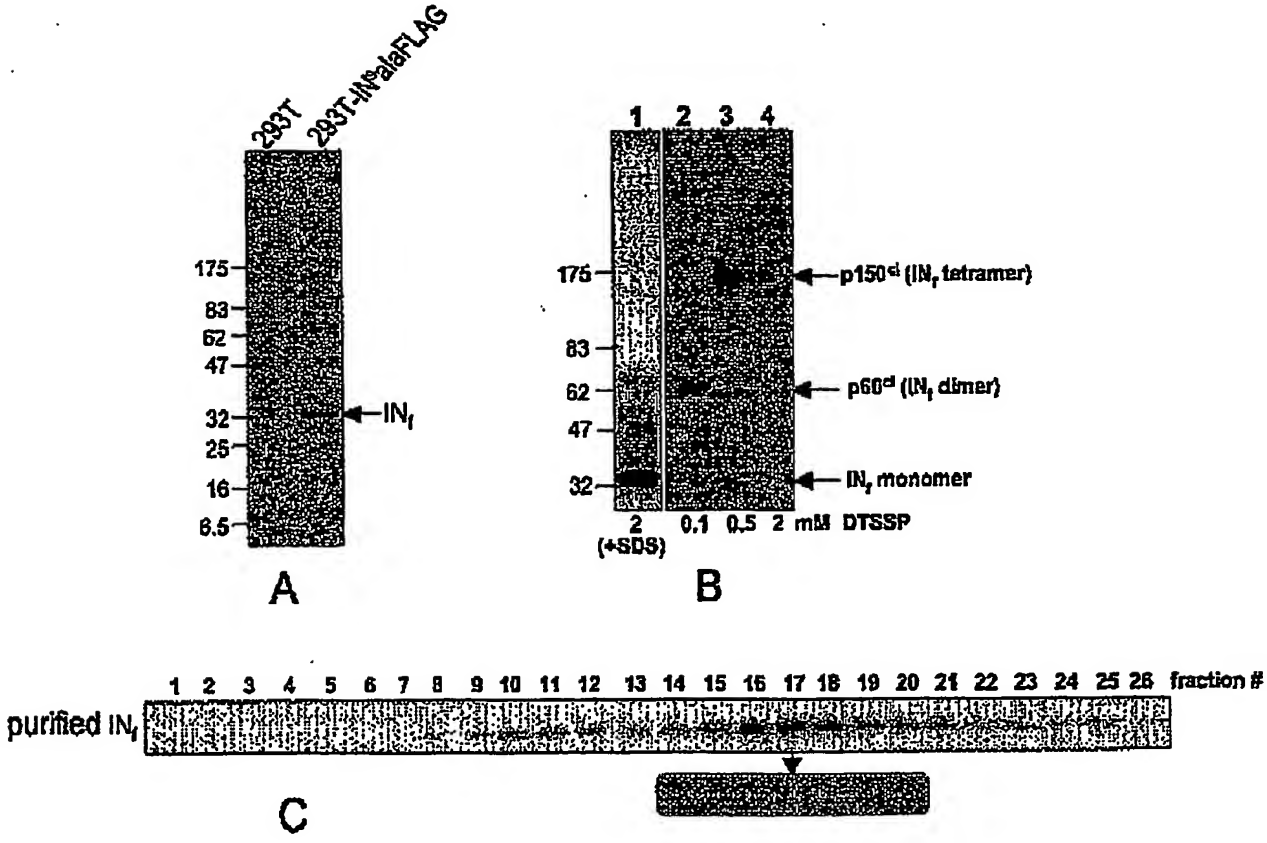
FIG. 4



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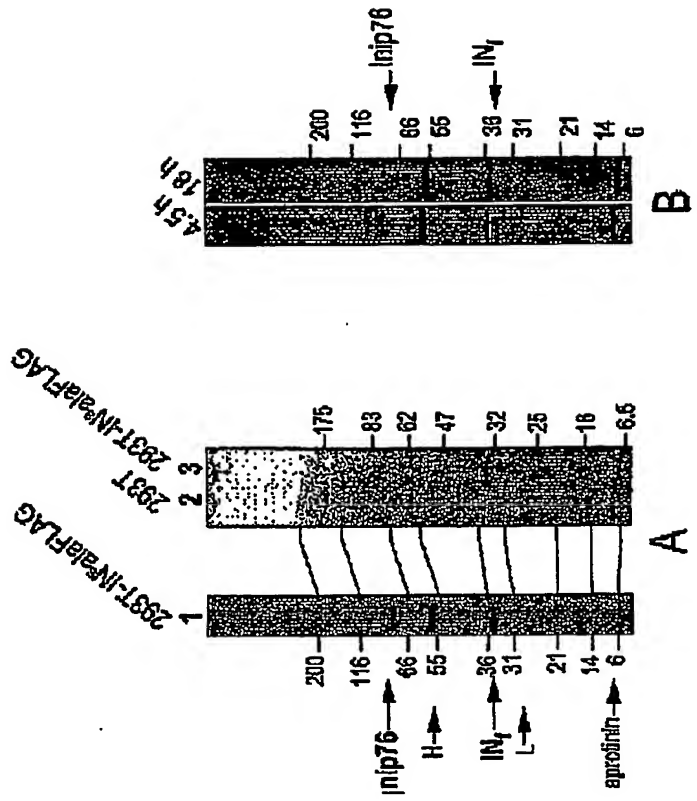

FIG. 5

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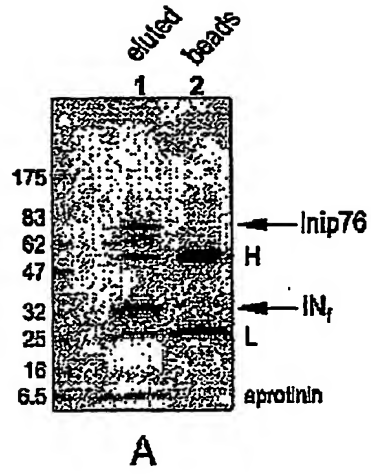
FIG. 6



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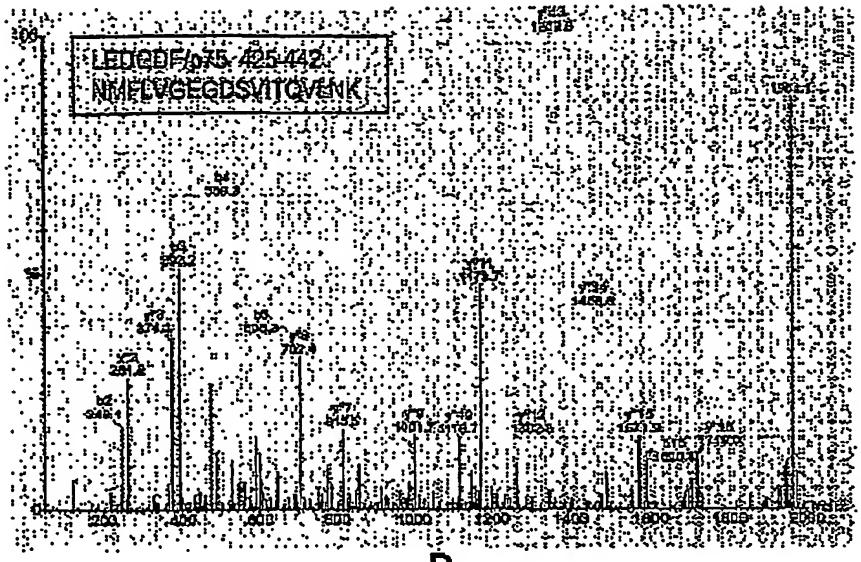

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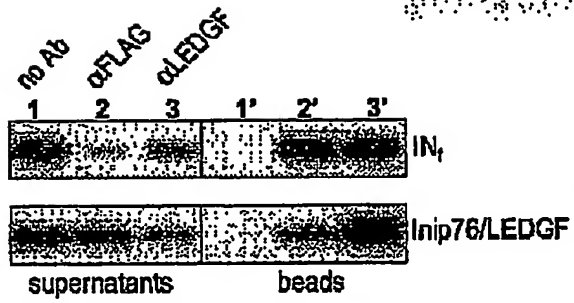

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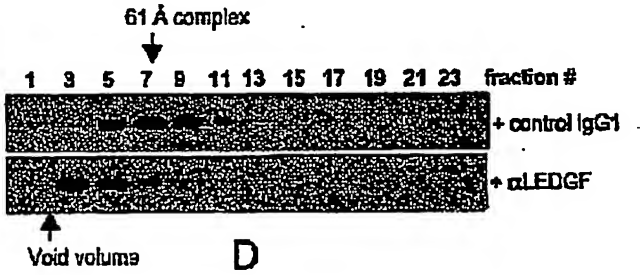

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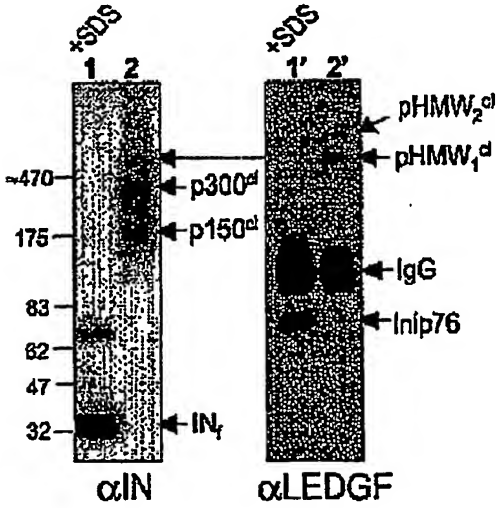

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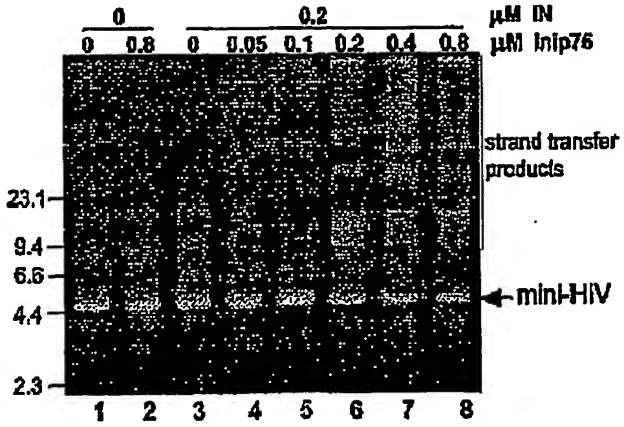

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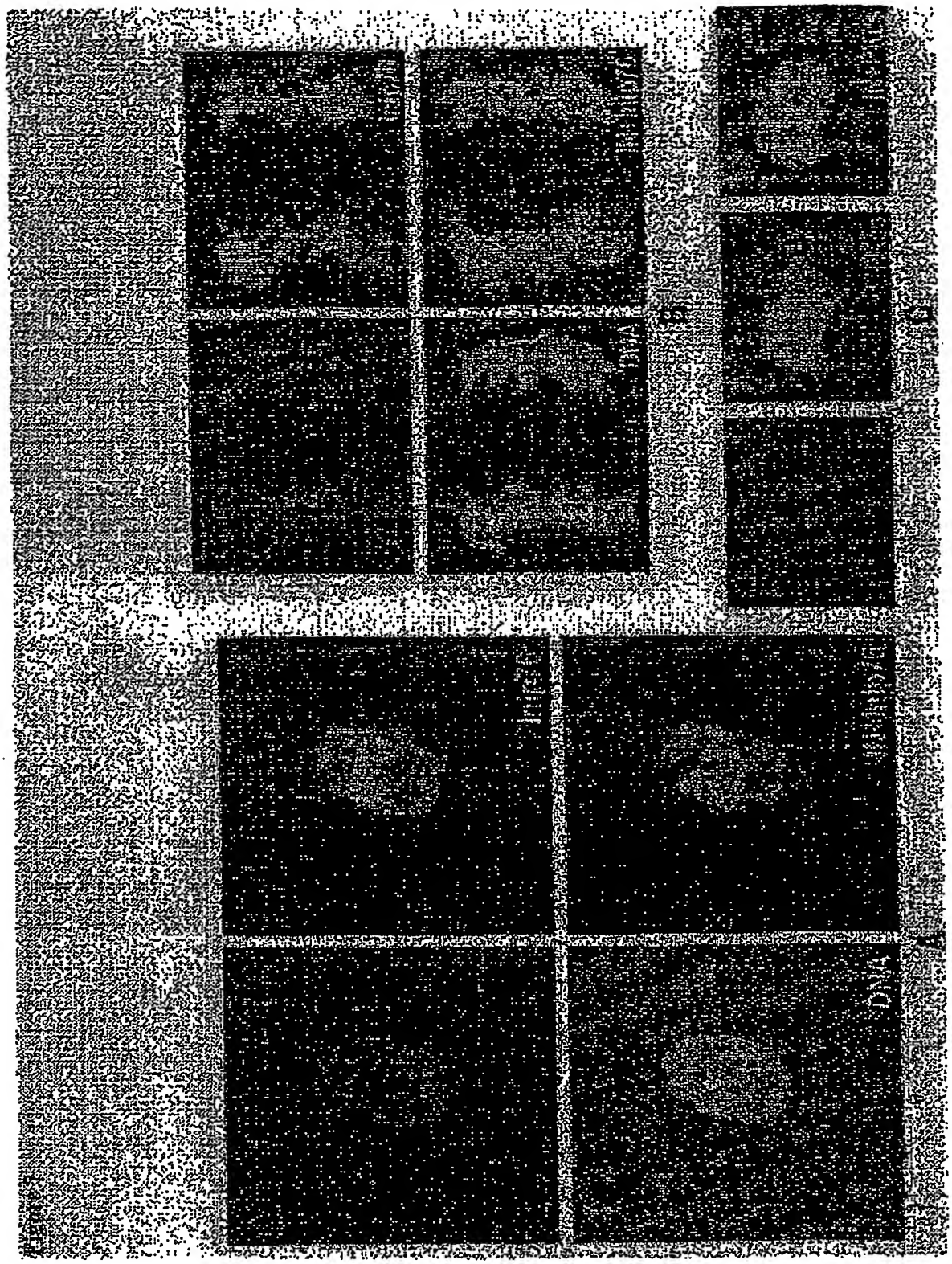

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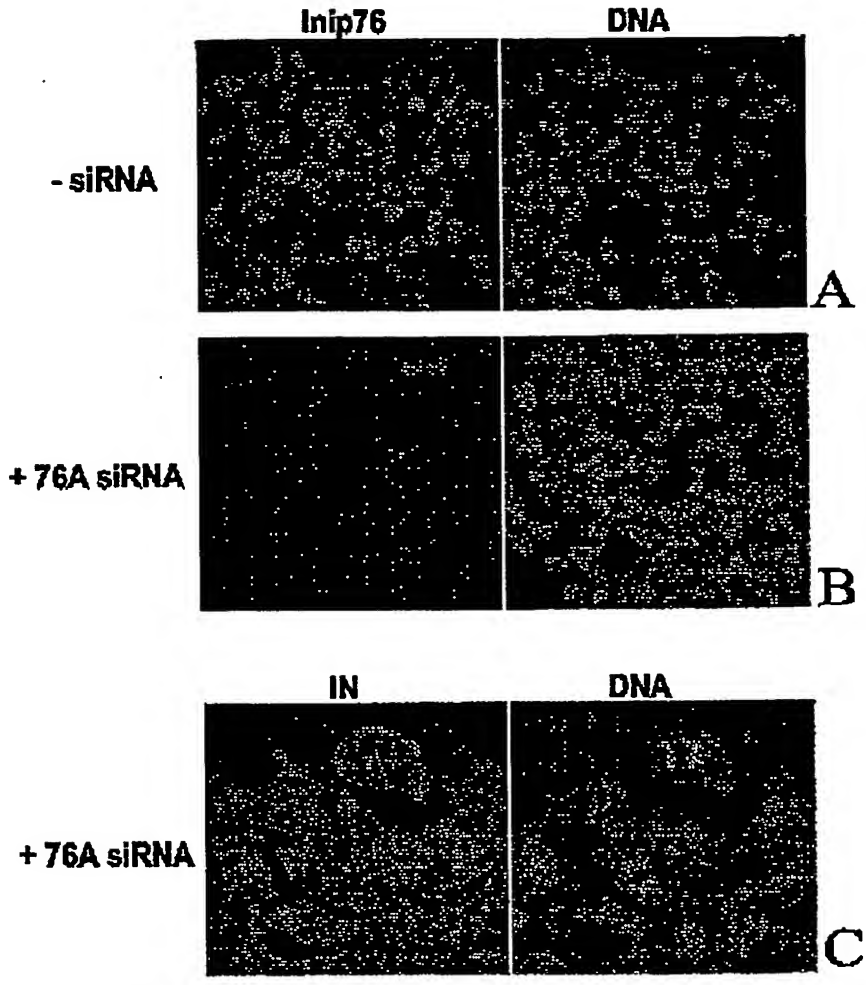
FIG. 8



siRNA mediated knock-down of the Inip76 expression and intracellular re-distribution of IN

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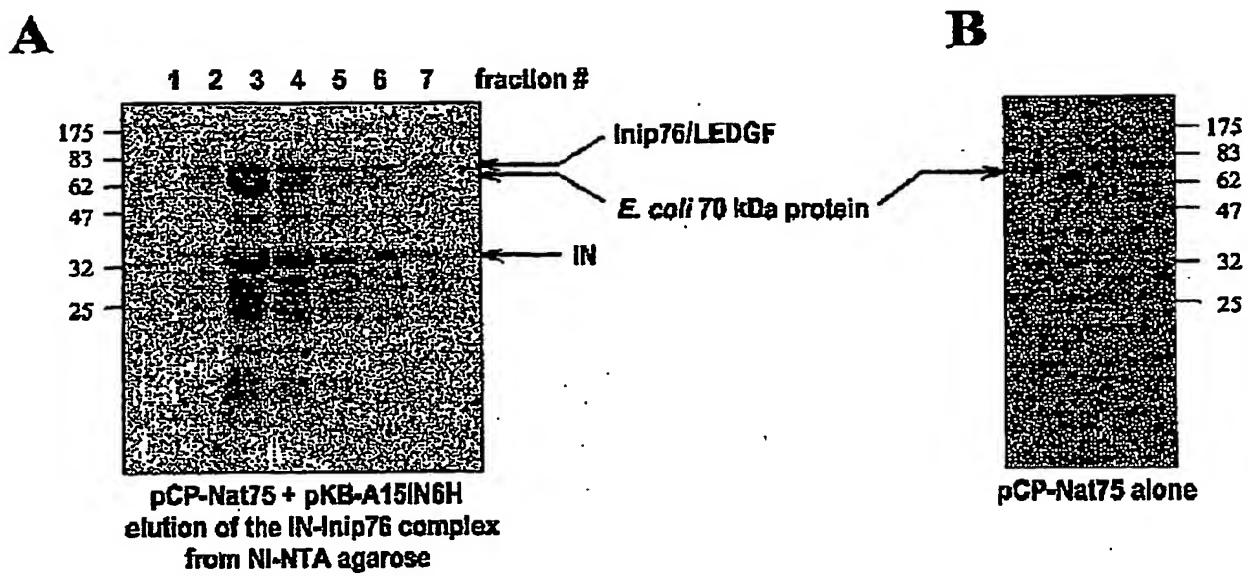
FIG. 9



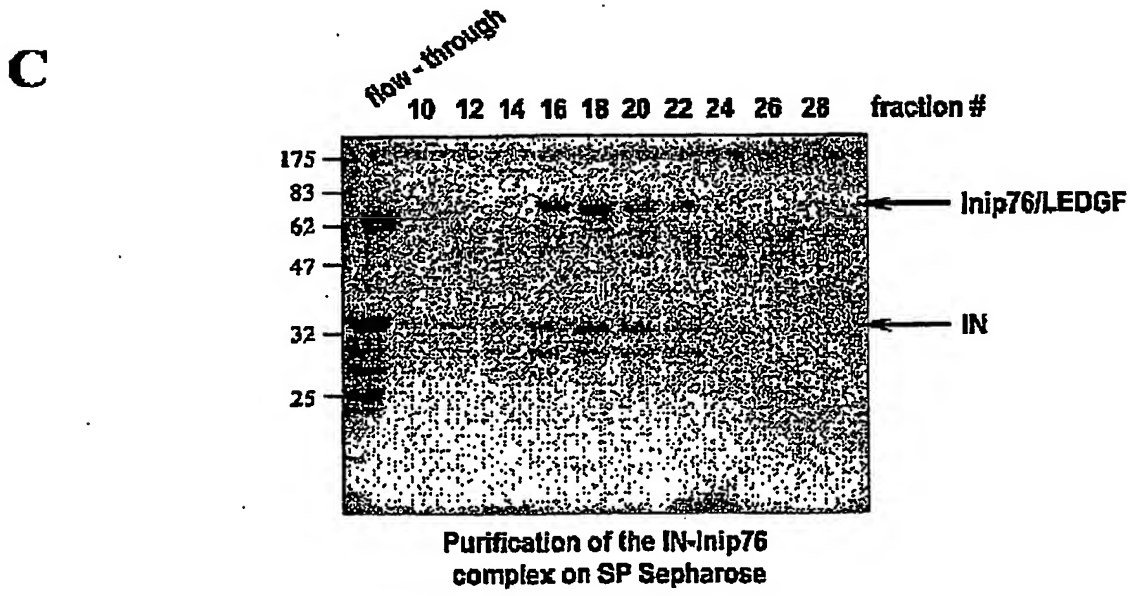


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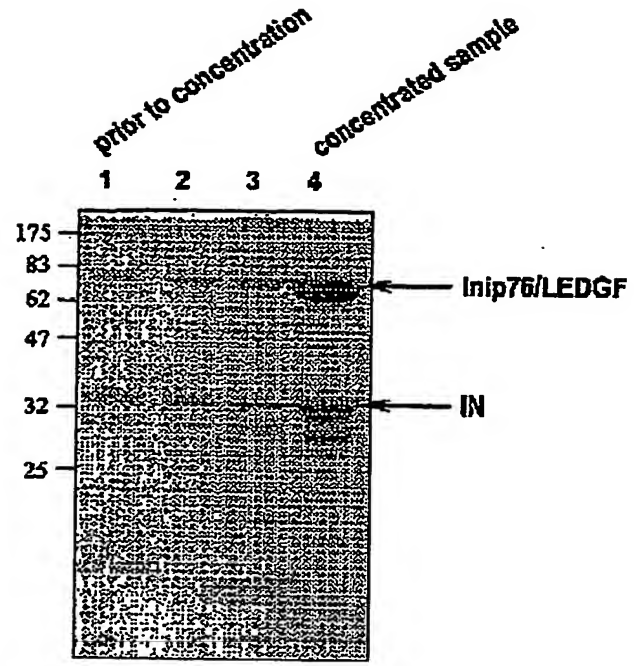
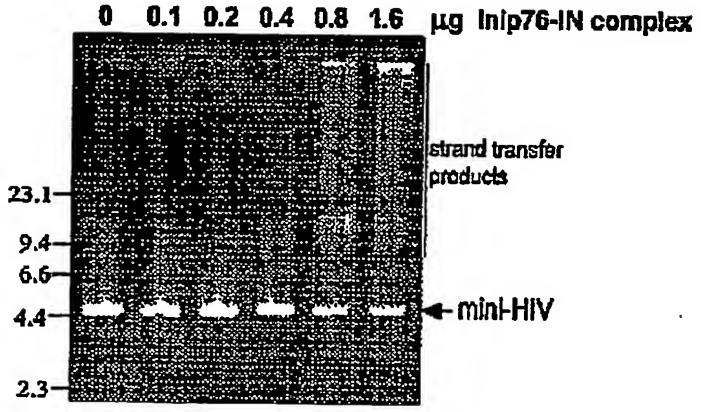
FIG. 10

A



Concentration of the IN-lnip76 complex

B



Activity of the isolated recombinant IN-lnip76 complex

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PCT Application

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